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Recent advances in design of antimicrobial peptides and polypeptides toward clinical translation

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ABSTRACT

The recent outbreaks of infectious diseases caused by multidrug-resistant pathogens have sounded a piercing alarm for the need of new effective antimicrobial agents to guard public health. Among different types of candidates, antimicrobial peptides (AMPs) and the synthetic mimics of AMPs (SMAMPs) have attracted significant enthusiasm in the past thirty years, due to their unique membrane-active antimicrobial mechanism and broad-spectrum antimicrobial activity. The extensive research has brought many drug candidates into clinical and pre-clinical development. Despite tremendous progresses have been made, several major challenges inherent to current design strategies have slowed down the clinical translational development of AMPs and SMAMPs. However, these challenges also triggered many efforts to redesign and repurpose AMPs. In this review, we will first give an overview on AMPs and their synthetic mimics, and then discuss the current status of their clinical translation. Finally, the recent advances in redesign and repurposing AMPs and SMAMPs are highlighted.

1. Introduction

Antibiotic resistance has become one of the major threats to public health. According to the CDC's 2019 threat report, more than 2.8 million people in the United States are infected with antibiotic-resistant bacteria each year and more than 35,000 people die as a direct result of these infections [1]. The global death toll caused by the antibiotic-resistant pathogens is projected to be 10 million each year by 2050, as warned by the World Health Organization in 2019 [2]. Many mechanisms of antibiotic resistance have been reported [3]. The majority of conventional antibiotics inhibit or kill bacteria by binding to their targets *via* a site-specific binding mechanism and thus imposing pressure on bacterial metabolism and proliferation [4,5]. However, this site-specific binding mechanism is also subjected to the rapid development of antibiotic resistance, as a simple mutation in the binding sites or modification on the structure of antibiotics could deactivate antibiotics [6,7]. Moreover, because many antibiotics act on intracellular targets, decreased membrane permeability and increased efflux pump activity are also important mechanisms of drug-resistance [8]. New potent antimicrobials that act through different mechanisms are in urgent need to counter the widespread antibiotic resistance. This need is especially stringent when many major pharmaceutical companies like Novartis have recently dropped their antibiotic research department due to unsatisfied profit. Furthermore, the recent outbreak of a multidrug-resistant *Candida auris* in 2019 and the ongoing pandemic of SARS-CoV-2 in 2020 further underscore the importance of maintaining an effective armoury of antimicrobial drugs to protect public health.

In the process of searching for new generation of antibiotics, antimicrobial peptides (AMPs) have received significant attentions during the

Abbreviations: AMPs, antimicrobial peptides; SMAMPs, synthetic mimics of AMPs; HDPs, host defence peptides; CDC, the Centers for Disease Control and Prevention; NCA, *N*-carboxyanhydride; ROP, ring-opening polymerization; SAR, structure-activity relationship; RAFT, reversible-addition fragmentation chain-transfer; ATRP, atom transfer radical polymerization; HC_{50}, the minimum concentration to lyse 50% of blood cell; MIC, the minimum concentration to completely inhibit microbial growth; MBC, the minimum concentration to kill 99.9% of microbes; LPS, lipopolysaccharide; PK, pharmacokinetics; PD, pharmacodynamics; SEC, size exclusion chromatography; FA, facially amphiphilic; RA, radially amphiphilic; MRSA, methicillin-resistant *Staphylococcus aureus*; VRE, vancomycin-resistant *Enterococcus*.

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past three decades. AMPs have coexisted with microbes for millions of years, but widespread resistance has not been reported, a strong indication that they have a unique antimicrobial mechanism that may evade the development of drug resistance. Unlike most of conventional antibiotics, AMPs kill or inhibit bacteria primarily through a membrane-active mechanism that involves neither site-specific binding nor interference of bacterial metabolism [9,10]. Despite several mechanisms of resistance have been reported [11], this membrane-active antimicrobial mechanism is still very difficult and expensive for bacteria to develop drug resistance [10]. Some AMPs have also been reported to display a broad range of biological activities against bacteria, fungi, parasites, insects, viruses and even cancer cells [12]. Consequently, AMPs and the synthetic mimics of AMPs (SMAMPs) have been extensively studied in the past 30 years as a new generation of antibiotics. However, the clinical translational development has achieved limited success so far, in part due to the high toxicity, low *in vivo* efficacy and poor enzyme stability of AMPs designed by current strategies. This review serves as an update on the recent development of AMPs and SMAMPs. Instead of inclusively discussing thousands of AMPs/SMAMPs reported so far, we focus only on the design strategies aiming to address the translational challenges encountered by current AMPs and SMAMPs. This review starts with an introduction on AMPs and their antimicrobial mechanisms, followed by a summary on the structure-activity studies of various synthetic mimics. The AMPs approved for clinical application and these under clinical trials, as well as the challenges in the translational development are then discussed. Finally, recent advances in redesign and repurposing AMPs/SMAMPs to improve their performance and translational potential are highlighted.

2. Antimicrobial peptides and their mechanisms of action

AMPs are a group of short peptides widely distributed in nature. They are synthesized either by the non-ribosomal or ribosomal pathways [13]. Non-ribosomally synthesized AMPs are found in bacteria and fungi. These AMPs are assembled by peptide synthetases. Examples include gramicidin, bacitracin and polymyxin B. In contrast, ribosomally synthesized AMPs, such as lanthipeptides, linaridins, and host defence peptides (HDPs), are gene-encoded peptides consisting of 12–50 amino acids with very little genetic overlap [14]. They are produced by a diverse range of species, from prokaryotes to humans. Extensive post-translationally-modifications give these AMPs a highly diverse structure and various biological activities [14]. This section gives an overview on the diversity and similarity of AMPs, and the well-recognized models of antimicrobial mechanisms.

2.1. Diversity and similarity of antimicrobial peptides

AMPs are found ubiquitously in all forms of life, ranging from bacteria to plants, fish, amphibians, insects and mammals [10]. They are part of the ancient, nonspecific innate immune system that defends the majority of living organisms during the initial stages of an infection [15,16]. So far, a total of 3197 antimicrobial peptides have been recorded in the Antimicrobial Peptide Database [17], of which 2374 peptides are from animals, 352 from plants, 356 from bacteria, 20 from fungi, and 13 from archaea and protists [17]. The diversity of antimicrobial peptides discovered so far is so huge that it is difficult to categorize them univocally, except broadly on the basis of their secondary structure (Fig. 1). AMPs are classified into four families based on their secondary structures: α-helix, β-sheet, combined α-helix and β-sheet (αβ), and non-αβ [18]. Among all the 3197 AMPs, 1911 (59.8%) peptides have unknown secondary structure. For these with known secondary structure, 451 (14.1%) peptides are α-helixes, 86 (2.7%) form β-sheets, 113 (3.5%) adopt αβ-conformation and 636 (19.9%) are non-αβ [17]. Most AMPs (97%) contain 12–50 residues, with an average length of 28 residues [12]. The majority of peptides (96%) have a net positive charge and the average net charge of all peptides is +4.6 [12]. AMPs are reported

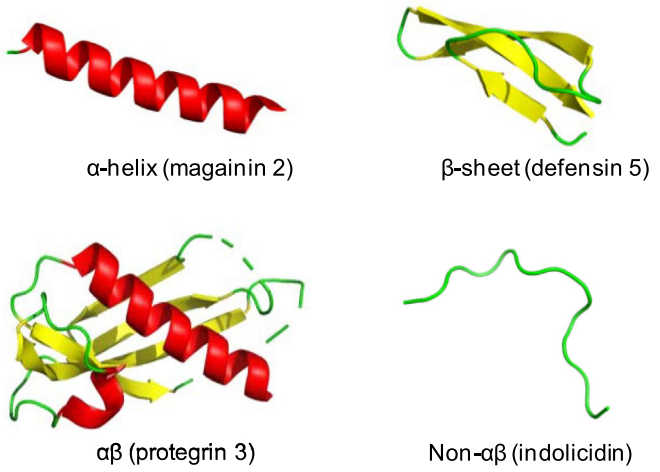


Fig. 1. Secondary structures of AMPs: α-helix (magainin 2, PDB: 4MGP), β-sheet (human defensin 5, PDB: 3I5W), combined α-helix and β-sheet (αβ, protegrin 3, PDB: 1KWI), and non-alphabeta (non-αβ, indolicidin, PDB: 1G89).

to have many biological activities [19]. Most AMPs (2680 peptides, 83.7%) have broad spectrum antibacterial activity against both Gram-negative and Gram-positive bacteria, several typical examples include magainin 2 [20], indolicidin [21], protegrin [22], human β-defensin-3 [23]. A significant portion of AMPs (1155 peptides, 36.1%) were reported to have anti-fungi activity. Dermaseptin, for example, was reported to actively kill yeast *Candida albicans* [24]. Others (190 peptides, 5.9%) have been reported to have anti-viral activity, such as maximin 1 [25], protegrin [22], and antiviral protein Y3 [26]. In addition to the well-recognized antimicrobial activities against bacteria, fungi and viruses, many AMPs were also reported to possess other activities. Such peptides include anticancer alloferon 1 [27], antiparasitic scorpine [28], anti-insect ponericins [29], and anti-inflammatory cathelicidin-PP [30]. In this review, we only focus on the antimicrobial activities, as the other activities are out the scope of this review.

Despite the tremendous diversity among various AMPs, most of them share two common features: an amphiphilic structure and a net positive charge [10]. AMPs generally contain both cationic and hydrophobic amino acid residues that can segregate into hydrophobic or cationic patches upon absorbing to bacterial membrane. This structural similarity is directly associated with their membrane-active antimicrobial mechanism.

2.2. Antimicrobial mechanisms

As part of the ancient innate immune system and the first line of defence, many AMPs have evolved to be able to selectively kill bacteria, with minimized toxicity to host. This selectivity is attributed to the fundamental differences in the cell surface structure between prokaryotic microbes and eukaryotic cells (Fig. 2a) [31,32]. For Gram-negative bacteria, their outer membrane is organized in such a way that the outermost leaflet of the lipid bilayer contains a high percentage of lipids with negatively charged head groups, examples include lipopolysaccharide (LPS) and phosphatidylglycerol [10,33]. Despite such an outer membrane is absent in Gram-positive bacteria, a thick layer of peptidoglycan cell wall decorated with negatively charged teichoic acids is covering the inner membrane of Gram-positive bacteria [33]. Meanwhile, the inner membrane of Gram-positive bacteria contains a higher percentage of negatively charged lipids than Gram-negative bacteria (40–50% *vs* ~20%, respectively) [34,35]. In contrast, the outer leaflet of mammalian cell membrane is principally composed of lipids with zero net charge, such as phosphatidylcholine and cholesterol [35]; most of the negatively charged lipids, like phosphatidylserine, are segregated

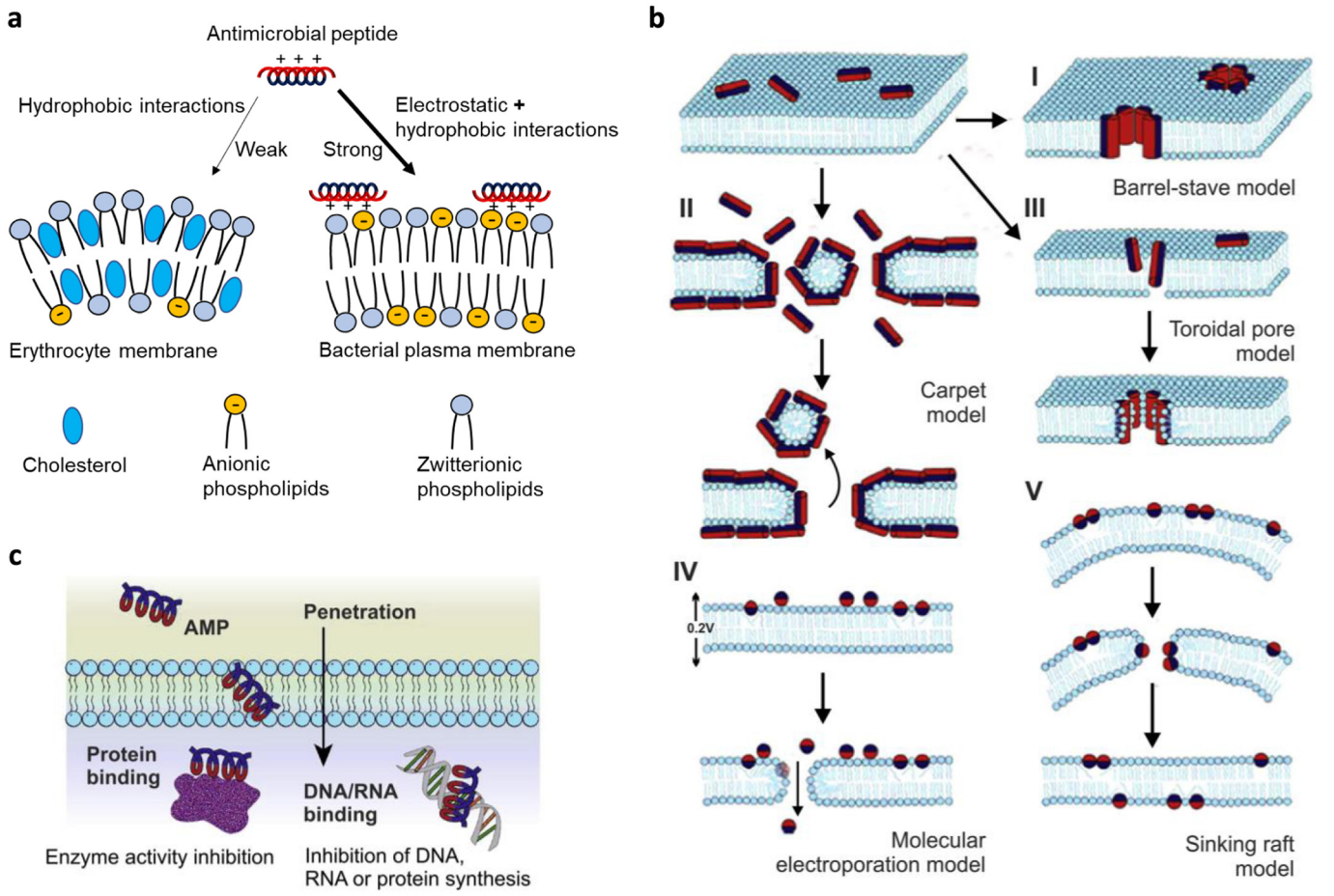


Fig. 2. Antimicrobial mechanisms of AMPs. (a) Selection between bacterial and mammalian cells is achieved by recognizing the difference in cell surface structure. Adapted with permission from [32]. Copyright 1999 Elsevier. (b) Models of membrane penetration. Adapted with permission from [41]. Copyright 2006 Elsevier. (c) Other antimicrobial mechanisms of AMPs. Reprinted with permission from [42]. Copyright 2017 Elsevier.

into the inner leaflet [10]. Therefore, AMPs preferentially absorb to the bacterial cells *via* electrostatic attraction, followed by insertion of their hydrophobic domains into the lipid bilayers to disrupt bacterial membrane. In addition to the difference in surface charge, the curvature of lipids also contributes significantly to the selectivity. In generally, mammalian membranes mainly contain lipids (phosphatidylcholine) with zero curvature, in which the lipid heads have a similar size to their lipid tails. On the other hand, bacterial membranes are rich in lipids with negative curvature (phosphatidylethanolamine and cardiolipin, 50–80%), whose lipid heads are smaller than their lipid tails [36]. It has been demonstrated by Wong and co-workers that these negative curvature lipids facilitate the formation of saddle-splay ("negative Gaussian") membrane curvature, which is a necessary condition for processes such as pore formation, blebbing, budding, and vesicularization, all of which destabilize the barrier function of cell membranes [37–40].

Although other antimicrobial mechanisms of AMPs have been reported [35,43], the most recognized one is membrane disruption. Several models of membrane permeation were proposed (Fig. 2b), including the three classic models: the barrel-stave, the carpet and the toroidal pore models [35,42,44]. All these models assume that AMPs adopt a facially amphiphilic (FA) conformation where the cationic and hydrophilic face is separated from the hydrophobic face upon absorbing to bacterial membrane. The barrel-stave model describes that the inserted FA-AMPs use their hydrophobic face to interact with the hydrophobic lipid tails, so that an aqueous pore across the lipid bilayer is encircled by the other hydrophilic face of AMPs [44]. In the toroidal model, the simultaneous interaction of both hydrophobic and hydrophilic faces of AMPs with the lipid tail and lipid head induces the lipid monolayers to bend continuously through the pore so that the water core is lined by both the AMPs and the lipid head groups [35]. In the carpet model, the AMPs orient parallelly to the surface of the lipid layer and disintegrate the lipid bilayer by forming micelle-like particles [42]. Other pore formation models have also been proposed, including the molecular electroporation model and the sinking raft model. In the molecular electroporation model, the accumulation of cationic AMPs on the outer membrane triggers the formation of nanopores *via* electroporation [45]. Molecular electroporation only occurs when the peptides present a sufficient charge density so that the electrostatic potential across the membrane is at least 0.2 V [45]. In the sinking raft model, the aggregation of AMPs on the membrane outer leaflet produces a mass imbalance across the membrane, which creates a curvature gradient that enables the peptides to sink into the membrane and creates a transient pore for the leakage of intracellular contents [46,47].

In addition to the membrane-active antimicrobial mechanism, another important mechanism to defend host is *via* immunomodulation. This is especially true in the multicellular organisms with developed immune systems. In fact, many HDPs released from the epithelia cells and neutrophils at sites of infection have a concentration range that is too low to have direct antimicrobial activities [48,49]. Yet they can still protect the host *via* immunomodulation that involves both innate and adaptive immune systems. The action of HDPs on innate immune cells which include monocytes, macrophages, dendritic cells, neutrophils, and natural killer cells is very complex, as they can interact with different receptors that are located either on the cell surface (Toll-like

receptors for example) or cytosol (NOD-like receptors) and initiate a variety of signalling pathways [50]. It is therefore expected that HDPs can influence a diverse range of innate immune responses. For example, LL-37 can modulate the inflammatory responses in macrophages, epithelial cells, peripheral blood mononuclear cells (PBMCs), and whole blood leukocytes by attenuating the Toll-like receptor responses, and modulating mitogen-activated protein kinase (MAPK) pathways and responses of tumor necrosis factor (TNF) and interleukin (IL) [48,51]. On the other hand, HDPs have also been shown to play multiple roles in adaptive immune responses such as promoting adjuvant responses to enhance adaptive immunity by directing immune functions toward responses regulated by helper T-cells (Th1 and Th2) [48]. For example, it was found that cells over-expressing defensins promote a strong Th1 response and induce the proliferation of cytotoxic T-cells and natural killer cells, and the production of IL-12 and interferon-γ in mice [52]. The immunomodulation activity allows HDPs to play important roles in various infectious diseases, inflammations and cancers. A comprehensive discussion on how HDPs modulate immune systems is out of the scope of this review. Readers are referred to several reviews and chapters specialized on the immunomodulation role of AMPs for more details [48,50,53].

Recent studies have provided increasing evidence that AMPs can also act on other bacterial targets as complementary antimicrobial mechanisms (Fig. 2c) [35,43]. Some AMPs, buforin II and magainin II for example, were reported to bind to DNA and interfere with DNA replication [54,55]. Other AMPs, such as hexapeptide WRWYCR, pleurocidin and indolicidin, were shown to inhibit the activity of enzymes that involve in DNA reparation [56], or RNA and protein synthesis [57,58]. Cell wall synthesis inhibition was also reported to be the antimicrobial mechanism of some AMPs such as nisin Z and plectasin [59,60].

It is important to note that AMPs do not rely on a single mechanism to protect the host. Instead, multiple mechanisms are usually involved and activated during an infection. The collaboration and synergy of multi-action also account for their low rate of resistance despite they have coexisted with bacteria for millions of years.

3. Synthetic mimics of antimicrobial peptides

The clinical application of natural AMPs is hurdled by several major challenges including toxicity, low-to-moderate *in vivo* efficacy, high cost for the development and production, and low protease stability. Synthetic approaches were adopted to optimize the activity, safety and stability of AMPs as well as to further understand their structure-activity relationship (SAR). In the last decade of 20th century, researchers started to use natural AMPs as the templates to optimize their activity and stability by mutating one or more amino acid residues. As better understanding on their SAR was developed, *de novo* design of a variety of synthetic peptides, peptoids, peptidomimetics, oligomers and polymers were followed in the first decade of 21st century. These studies significantly deepened our understanding on the SAR of AMPs and also developed many promising drug candidates for clinical trials.

3.1. Synthetic antimicrobial peptides, peptoids and peptidomimetics

Antimicrobial peptides represent a class of promising therapeutic agents against bacteria, fungi and viruses. However, most natural AMPs have suboptimal activity, safety and stability for clinical application and further optimization is usually needed. The most straightforward approach is to use the natural AMPs as the templates and mutate one or more amino acid residues to other proteinogenic L-residues for achieving enhanced antimicrobial activity and selectivity. For example, pexiganan (GIGKFLKKAKKFGKAFVKILKK) is a synthetic mimic of natural AMP magainin 2 (GIGKFLHSAKKFGKAFVGEIMNS). With some of the neutral and anionic amino acid residues being replaced by cationic or hydrophobic amino acid residues, pexiganan exhibits potent broad spectrum antimicrobial activity against both Gram-negative and Gram-positive bacteria [61,62]. In fact, several other synthetic AMPs entered the late stages of clinical trials were developed by this method. A few examples include iseganan, omiganan, and P113, which were developed from protegrin, indolicidin and histatin, respectively [63–65]. The AMPs approved by FDA and these under clinical trials will be further discussed in Section 4.

One important factor limiting the clinical application of natural AMPs is their protease instability. It has been demonstrated that some AMPs can be readily degraded by protease [66,67]. One potential solution is to replace the proteinogenic L-amino acids with D-amino acids that are generally more resistant to protease [68,69]. Merrifield and co-workers replaced all the L-amino acids in cecropin A, magainin 2 and melittin with D-amino acids [69]. All the D-enantiomers exhibit similar antimicrobial activity to their natural counterparts but are less toxic. More importantly, they are much more resistant to enzymatic degradation. Shai and co-workers synthesized diastereomers of the bee venom melittin by replacing some of L-amino acid in natural melittin with D-amino acid [70]. The melittin diastereomers retain the antimicrobial activity, but the cytotoxicity is significantly decreased. This is because the diastereomers only bind to negatively charged lipid, while the native melittin binds to both negatively charged and zwitterionic lipids that are rich in human cell membrane. In addition to linear AMPs, cyclic AMPs were also demonstrated to have excellent protease stability. Ghadiri and co-workers developed antimicrobial peptides based on cyclic D, L-α-peptides with 6–8 residues (Fig. 3a) [71,72]. Those cyclic peptides are protease-resistant and have broad antibacterial spectrum. Interestingly, these cyclic peptides have planar conformation and can form nanochannels on bacterial membrane by stacking on top of each other *via* hydrogen bonding. These nano-channels depolarize the membrane potential to kill bacteria. Another method to synthesize protease-stable AMPs is to utilize β-amino acids as the building blocks [73–75]. Gellman and co-workers synthesized helical β-peptides from β-amino acids [74,75]. These β-peptides adopt a helical conformation with about 2.5 residues per turn that is distinct from the natural α-helices (Fig. 3b). They have higher antibacterial activity than magainin and are more resistant to protease degradation. Additionally, some groups synthesized peptoids and peptidomimetics using non-natural *N*-substituted amino acids [76–80]. Barron et al. synthesized oligo-*N*-substituted-glycine-based helical peptoids to mimic magainin 2 amide (Fig. 3c) [77,78]. These peptoids exhibit good antibacterial activity and selectivity that are comparable to magainin 2. They also have good protease resistance. Cai and co-workers developed a series of peptidomimetics with or without lipid tails based on *N*-acylated-*N*-aminoethyl amino acid residues (Fig. 3d) [80–82]. These peptidomimetics exhibit good protease stability and potent antimicrobial activity against Gram-positive bacteria and fungi.

Synthetic peptides and peptide mimics are generally more stable than natural AMPs and some of them possess better activity and selectivity. However, significant amount of labor and time are still required to produce them since stepwise synthesis is usually required. Eventually, the high cost becomes one of the major factors limiting their application. Nevertheless, through the above work, a better understanding in the SAR of AMPs was developed. The fact that the replacement of some amino acid residues in AMPs with other residues does not necessarily cause the loss of antimicrobial activity but sometimes even enhance it indicates that the specific amino acid type is not required for antimicrobial activity. Moreover, as peptoids and peptidomimetics with simple repeating sequence also have high antimicrobial activity, specific amino acid sequence was also ruled out to be a necessity for antimicrobial activity. Therefore, it was recognized that the activity and toxicity of AMPs are determined primarily by the overall physicochemical properties, less by the specific amino acid type and sequence.

3.2. Antimicrobial oligomers and polymers

The pioneer studies in synthetic antimicrobial peptides, peptoids and peptidomimetics suggest that it is the overall physicochemical

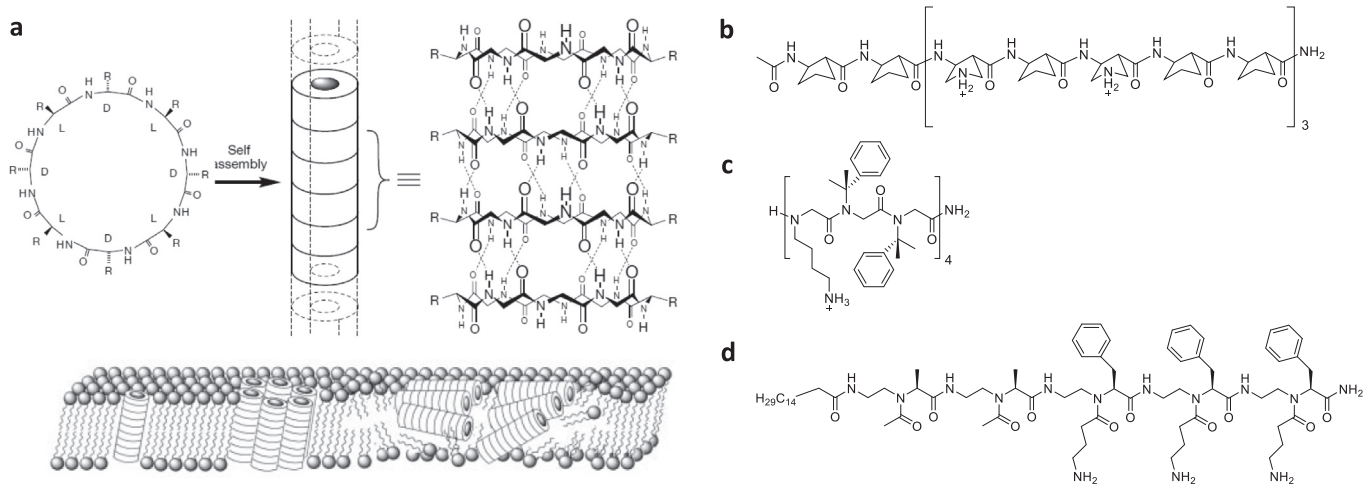


Fig. 3. Structure of representative synthetic peptides, peptoids and peptidomimetics. (a) Structure of a cyclic $_{D,L}$-α-peptide and its self-assembly in membrane. The stacking of cyclic peptides forms nano-channels, through which the ions and cytoplasma contents leak out. Reprinted with permission from [71]. Copyright 2001 Springer Nature. (b) Structure of a helical β-peptide synthesized from β-amino acids. (c) Structure of a peptoid synthesized from *N*-substituted glycine. (d) Structure of a lipidated peptidomimetic synthesized from *N*-acylated-*N*-aminoethyl amino acids.

properties, especially the amphiphilicity and cationic charge, that determine the antimicrobial activity and cytotoxicity of AMPs. Researchers then turned to the *de novo* design of synthetic antimicrobial oligomers and polymers by mimicking these two key physicochemical properties. In this work, we define polydispersed molecules with molecular weights (Mw) of 1000–3000 Da as oligomers and these with Mw larger than 3000 Da as polymers. Monodispersed antimicrobial molecules with Mw less than 1000 Da are defined as small SMAMPs, which is a class of promising antibiotic candidates and will be further discussed in Section 6.4. Synthetic antimicrobial oligomers and polymers can be synthesized by facile polymerization methods including the living free radical polymerization methods (RAFT and ATRP) and the ring-opening polymerization (ROP) methods. The remarkable advantage of polymeric SMAMPs over the previously described peptide- or peptoid-based SMAMPs is that they can be obtained in large quantity in very few synthetic steps, whereas the peptides and peptoids require labor-intensive and expensive stepwise synthesis and typically cannot be obtained in large quantity. Meanwhile, like peptoids and peptidomimetics, the polymeric SMAMPs generally have good resistance to environmental stress and protease degradation due to their non-natural origin. Moreover, the availability of building blocks is also greatly expanded as many cheap and commercially available non-amino acid monomers can also be used to synthesize antimicrobial oligomers and polymers. Many different types of polymeric SMAMPs have been reported. A few examples include poly(methacrylate) derivatives [83–87], nylon-3 derivatives [88–91], poly(norbornene) derivatives [92,93], poly(phenylene ethynylene) derivatives [94–97], poly(4-vinylpyridine) derivatives [98] and poly(oxanorbornene) derivatives [99,100] (Fig. 4). Although the clinical translation of these polymeric SMAMPs is usually hampered by their heterogeneous nature and safety concerns, the SAR

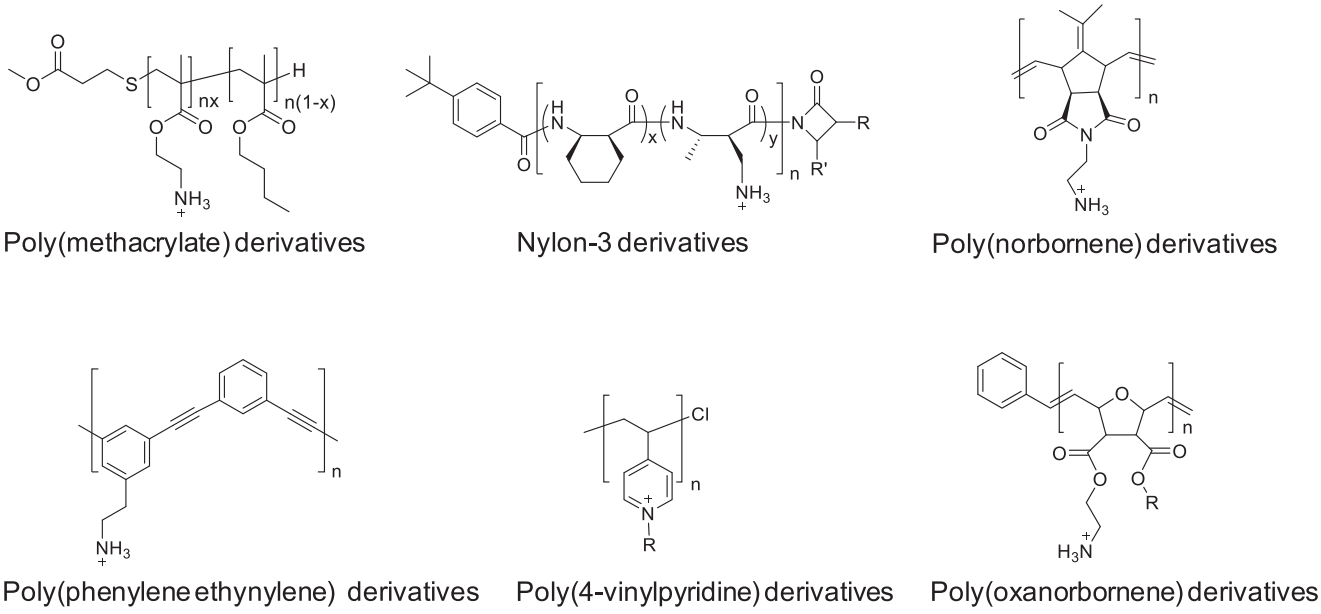


Fig. 4. Structure of representative antimicrobial polymers and oligomers based on poly(methacrylate), nylon-3, poly(norbornene), poly(phenylene ethynylene), poly(4-vinylpyridine), and poly(oxanorbornene).

of AMPs was further uncovered using these polymeric SMAMPs as models. Therefore, this section focuses on the discussion of the key parameters that determine the activity and toxicity of AMPs as uncovered by the structure-activity studies of polymeric SMAMPs.

3.2.1. Amphiphilicity

Amphiphilicity is one of the most important factors that determines the antimicrobial activity and selectivity of AMPs and is also the most extensively studied and discussed. It is recognized that a balanced amphiphilicity is required to reach the optimal activity and selectivity. However, this "balance" has never been quantified and varies by different types of materials. Therefore, fine-tuning is usually needed to approach this "balance". In early works, Kuroda and Degrado synthesized a series of random copolymers based on poly(methacrylate) and used them as model compounds to demonstrate that even a non-peptide system with random sequence can actively kill bacteria when the hydrophobic and cationic components are introduced into the structure [83]. Both antimicrobial activity and hemolytic toxicity initially increased with the increase of hydrophobicity, but an overly hydrophobic structure led to decreased activity and increased hemolysis. This series of polymers have low selectivity (HC_{50}/MIC <1) in killing bacteria over mammalian cells. In the follow-up work, they varied the polymer compositions to fine-tune the amphiphilicity and achieved enhanced activity and selectivity (HC_{50}/MIC up to 170) [84,85,87]. Gellman, Stahl, Masters, and co-workers also found that a "balanced amphiphilicty" is required to obtain the optimal antimicrobial activity and selectivity from the structure-activity studies of nylon-3 based amphiphilic and cationic polymers [88,90,91]. Interestingly, by tuning the amphiphilicity of nylon-3 based copolymers, they discovered several polymers with high activity (MIC as low as 3.1 μg/mL) against drug-resistant fungi and fungal biofilm [101–103]. Tew and co-workers synthesized a series of polynorbornene and poly(oxanorbornene) based facially amphiphilic polymers [99,100]. Unlike the random copolymers developed by Kuroda and Gellman, which bear cationic groups and hydrophobic groups on separated monomers, the facially amphiphilic polymers developed by Tew have cationic and hydrophobic groups on the same monomer (Fig. 4). This facially amphiphilic structure allows easier control and prediction of the amphiphilicity and conformation of polymers. Therefore, several facial amphiphilic polymers with high activity and selectivity (HC_{50}/MIC up to 500) were obtained.

3.2.2. Charge

Charge is another important determinant factor for the activity and toxicity of AMPs. Both the charge group type and the charge density were shown to significantly affect the antimicrobial activity and toxicity. Gellman and co-workers found that poly(4-dimethylaminomethyl styrene) derivatives with tertiary amine have higher antimicrobial activity and hemolysis than polymers with quaternary ammonium [104]. Kuroda and co-workers compared the antimicrobial activity of methacrylate based copolymers bearing primary, tertiary, or quaternary amine/ammonium as the cationic groups and found that copolymers with primary amine have the highest activity and hemolysis, followed by tertiary amine and then quaternary ammonium [84]. Guanidinium was also used as the cationic group and was reported to have enhanced antimicrobial activity over amines [105], possibly because it can generate both charge interaction and bidentate hydrogen bonding with lipids. Besides the chemical identity of the charged groups, the charge density has also been shown to be an activity determinant. Tew and co-workers investigated the effects of different charge density on antimicrobial activity by functionalizing the repeating units of a norbornene-based polymer with one, two, or three primary amine groups [93]. They found that polymers with higher charge density have alleviated hemolytic toxicity, but similar antimicrobial activity compared to these less charged polymers. Similarly, tuning the total number of cationic residues in AMPs has been reported as a strategy to optimize their antimicrobial activity [106].

3.2.3. Molecular weight

Molecular weight has also been shown to play a role in tuning the antimicrobial activity and selectivity, especially in regulating the specificity between Gram-negative and Gram-positive bacteria. For hemolysis, the majority of studies have shown that a higher molecular weight leads to higher hemolysis [83,87,99]. For antimicrobial activity, however, no simple correlation was found. Kuroda and Gellman reported that the antimicrobial activity of both polymethacrylate and nylon-3 based polymers slightly increases with the increase of molecular weight [83,90]. Tew and co-workers, however, found the otherwise [92]. An interesting observation reported by Tew and co-workers is that poly(oxanorbornene) based polymers of different molecular weight can differentiate Gram-negative and Gram-positive bacteria [99]. Oligomers (<3 kDa) have high activity against Gram-positive bacteria, but low activity against Gram-negative bacteria. Polymers (10 kDa), however, show the opposite trend. This antimicrobial specificity is possibly due to the filtering effect of the negatively charged peptidoglycan mesh of Gram-positive bacteria, which is more efficient in blocking the larger cationic polymers from reaching the plasma membrane compared to smaller oligomers. On the other hand, the larger polymers are more efficient in disrupting both the inner and outer membranes of Gram-negative bacteria.

The structure-activity studies of antimicrobial oligomers and polymers have revealed that the amphiphilicity, the identity and density of cationic groups, and the molecular weight all play important roles in determining the antimicrobial activity and selectivity of AMPs and SMAMPs. By fine-tuning these parameters, oligomers and polymers with high antimicrobial activity and safety have been reported.

3.3. Antimicrobial polypeptides synthesized from amino acid N-carboxyanhydrides (NCAs)

In addition to the non-amino acid-based antimicrobial polymers, another type of antimicrobial polymers based on amino acid building blocks, referred as antimicrobial polypeptides here, have also been reported. These polypeptides were synthesized by the ring-opening polymerization (ROP) of the amino acid NCAs (Fig. 5a) [107–114]. The reason why these polypeptides are separately discussed is because their unique characteristics do not simply fall into the definition of either peptides or polymers: Unlike conventional peptides, these polypeptides do not have a well-defined primary sequence, yet they have a peptide backbone. In other words, these polypeptides simultaneously have the advantages of both conventional peptides and polymers. On the one hand, they are biodegradable polymers with peptide backbones. By rational designing the structure of NCA monomers, antimicrobial polypeptides can have controllable secondary structures and good protease resistance [110–112]. On the other hand, similar to polymers, the NCA-ROP method allows the easy synthesis of antimicrobial polypeptides in very few steps and large quantity, without the need of stepwise synthesis that is required for the synthesis of sequence-defined peptides, peptoids and peptidomimetics.

A variety of antimicrobial polypeptides have been synthesized by NCA-ROP and their activity and toxicity were studied [107–117]. Park and coworkers evaluated the antimicrobial activity of random co-polypeptides synthesized by the ROP of lysine-NCA and the NCAs of alanine, phenylalaine and/or leucine (Fig. 5b). It was found that an optimal combination of hydrophilic and hydrophobic amino acids, namely a "balanced" amphiphilicty, is needed to reach the optimal antimicrobial activity and selectivity [109]. Hammond and co-workers synthesized a homo-polypeptide poly(γ-propargyl-$_L$-glutamate) *via* the NCA-ROP, and used it as a platform to attach a variety of functionalities, including primary, secondary, tertiary, and quaternary amines/ammonium with hydrocarbon side chains ranging from 1 to 12 carbons long

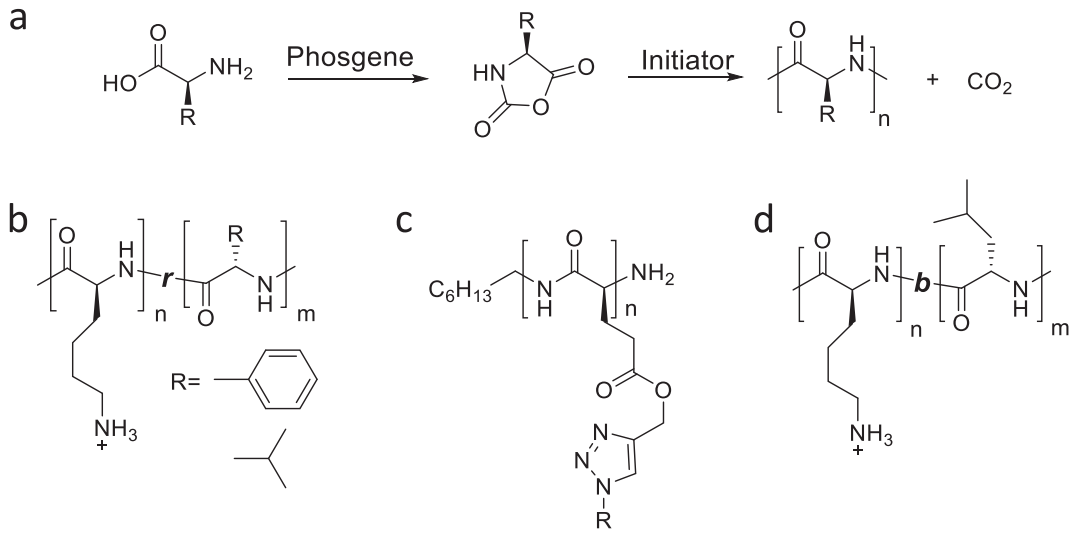


Fig. 5. Antimicrobial polypeptides synthesized from amino acid *N*-carboxyanhydride (NCA) by ring-opening polymerization (ROP). (a) A general scheme for the synthesis of polypeptides by NCA-ROP. (b) Structure of antimicrobial random co-polypeptides reported by Park [109]. (c) Structure of antimicrobial homo-polypeptides reported by Hammond [108]. (d) Structure of antimicrobial block co-polypeptides reported by Deming [107].

(Fig. 5c). They found that polypeptides with different amine groups have antimicrobial activity in the order of primary > secondary > tertiary ~ quaternary ammonium and that the antimicrobial activity increases with the increase of molecular weight [108]. These findings are consistent with Kuroda's work as discussed in Section 3.2. Moreover, they further confirmed that a balanced amphiphilicity ($R = C_8H_{17}$ in this case) can help reach the optimal antimicrobial activity and selectivity. Deming and co-workers reported a block co-polypeptide $K_{100}L_{40}$ synthesized by NCA-ROP (Fig. 5d). This block co-polypeptide forms a self-supporting hydrogel in water and provides an effective barrier to microbial contamination of wounds, as measured by multi-log decreases of tissue-associated bacteria with deliberate inoculation of porcine skin explants, porcine open wounds, and rodent closed wounds with foreign body [107].

4. Clinical application and development

The history of AMP development can be divided into two waves [118]. Prior to the 1980s, the first wave of AMP research has led to the discovery of several non-gene-encoded (non-ribosomal biosynthesis pathway) peptide antibiotics that have been used in clinic and food storage for several decades. The second wave started in the 1980s and continues to nowadays amid an era when the antibiotic resistance has become one of the major health threats to human society. The second wave mostly focuses on the discovery of gene-encoded (ribosomal biosynthesis pathway) AMPs and the SAR of their synthetic mimics [118]. Extensive efforts have also been put on their antimicrobial mechanisms as well as their role in innate immunity and modulation of the immune systems. Despite significant progresses have been made in the second wave of studies, most of the AMPs approved for clinical application and food storage are those discovered in the first wave.

4.1. AMPs approved for clinical application and food storage

Several AMPs have been approved for clinical application and food storage (Table 1). They vary in structure, antimicrobial mechanism and spectrum of activity. While the majority of them have a membrane-active antimicrobial mechanism, bacitracin kills bacteria by inhibiting cell wall biosynthesis [119,120]. Notably, most of them adopt a cyclic structure and are positively charged except for daptomycin, which is in fact negatively charged. However, it should be noted that daptomycin turns into active form only when it binds to calcium ions to reverse its charge state from anionic to cationic [121].

Table 1
Antimicrobial peptides approved for clinical application and food storage.

AMPs	Conformation	Indications/uses	Antimicrobial spectrum	Administration	Mechanism of action	Year of approval
Gramicidin D [122]	Linear helical peptides	Skin, eye and wound infections	Gram-positive bacteria	Topical	Forming ion channel-like pores in bacterial membrane	~1940
Gramicidin S [123,124,126]	Cyclic beta-sheet peptide	Wound infections, spermicide and genital ulcers	Gram-positive bacteria, some Gram-negative bacteria and fungi	Topical	Disruption of bacterial membrane	1942
Bacitracin [119,120]	Cyclic lipopeptide	Skin, eye and wound infections	Gram-positive bacteria	Topical	Interference of cell wall and peptidoglycan synthesis	1948
Nisin [59]	Polycyclic lantibiotics	Food preservative	Gram-positive and some Gram-negative bacteria	N.A	Disruption of bacterial membrane	1960
Polymyxin B [127–130]	Cyclic lipopeptide	Meningitis, pneumonia, sepsis, urinary tract infections	Gram-negative bacteria	Intravenous and inhale	Disruption of bacterial membrane	1964
Colistin [131]	Cyclic peptide	Cystic fibrosis, intestinal infections	Gram-negative bacteria	Intravenous, oral, inhale	Disruption of bacterial membrane	1970
Daptomycin [121,132]	Cyclic lipopeptide	Endocarditis, skin infections, bacteraemia,	Gram-positive bacteria	Topical and intravenous	Disruption of bacterial membrane	2003

Here we only discuss four representative AMPs approved for clinical application (Fig. 6). Gramicidin S (GS, primary structure cyclo(-Val-Orn-Leu-D-Phe-Pro-)$_2$) was first isolated from the Gram-positive bacterium *Bacillus brevis* and is one of the first AMPs used in clinic (first used in Soviet in 1942). Even though gramicidin S is a derivative of gramicidin D, they have very different structure. While gramicidin D is a mixture of three 15-mer peptides named gramicidin A, B and C and has a linear helical conformation [122], gramicidin S is a 10-mer cyclic peptide adopting a β-sheet conformation [123]. This cyclic β-sheet conformation has been demonstrated to be critical for the antimicrobial activity, as it elegantly separates the cationic and hydrophobic side chains into the opposite faces of the molecule, thereby facilitating its interaction with bacterial membrane [124]. Meanwhile, studies have shown that the antimicrobial activity of a linearized gramicidin S is drastically reduced compared to the native gramicidin S (MIC: 3 μg/mL for cyclic GS *vs* 120 μg/mL for linear GS against *S. aureus*) [125]. Gramicidin S actively kills both Gram-positive and Gram-negative bacteria by disrupting their bacterial membrane.

Bacitracin is cyclic peptide with zero net charge introduced into market in 1948. Unlike most AMPs that kill bacteria by disrupting bacterial membrane, bacitracin interrupts the dephosphorylation of C_{55}-isoprenyl pyrophosphate and bactoprenol pyrophosphate, both of these lipids function as carrier molecules that transport the building blocks of the peptidoglycan [119,120]. Therefore, bacitracin is selectively active toward Gram-positive bacteria. Upon introduction into clinical use, bacitracin was found to be nephrotoxic, and systemic use was soon withdrawn [133]. However, it continued to be used topically for wound and skin infection. In contrast to its low level of use in humans, bacitracin has been used extensively as a growth promoter in animal husbandry. However, potential cross-resistance to structurally related antibiotics is possible [134].

Polymyxin B is a cationic cyclic lipopeptide approved by FDA in 1964. It is a highly potent AMP that selectively kills Gram-negative bacteria by disrupting the bacterial membrane [129]. Its selectivity toward Gram-negative bacteria comes from its high affinity to LPS that is abundant in the outer membrane of Gram-negative bacteria [128,129]. Due to its high affinity to LPS, polymyxin B is also the most efficient compound for the treatment of septic shock. The lipid tail was demonstrated to be important for its membrane disruption activity. Without this lipid tail, it binds to LPS with high affinity but has an extremely low antibiotic effect [127].

Daptomycin is an anionic lipopeptide that selectively kills Gram-positive bacteria, which is the very opposite example of polymyxin B. The mechanism studies revealed that daptomycin first binds to Ca^{2+} in solution at 1:1 M ratio to reverse its negative charge to positive charge [121]. The positively charged daptomycin-Ca^{2+} complex then absorb to the negatively bacterial membrane *via* electrostatic interaction and forms multimers to disrupt bacterial membrane. The reason for the selectivity against Gram-positive bacteria is not clear. However, it is believed that the higher percentage of negatively charged lipids in the membrane of Gram-positive bacteria over Gram-negative bacteria accounts for the selectivity [132].

4.2. AMPs and SMAMPs under clinical trials

So far, numerous AMPs and SMAMPs developed in the second wave of AMP research have entered clinical trials and significant progresses have been achieved. A few examples are listed in Table 2. Overall, the majority of these drug candidates are derivatives of natural AMPs by mutating one or more amino acid residues (entry 1–12). In particular, delmitide is the D enantiomers of HLA class I by replacing all L-amino acid residues with D-amino acid residues to enhance the protease resistance (entry 12) [135]. Natural AMPs have also entered clinical trials (entry 13). Rationally designed synthetic peptides were also developed by giving them a cationic and amphiphilic structure. Some of rationally designed synthetic peptides have successfully entered phase IIb or even

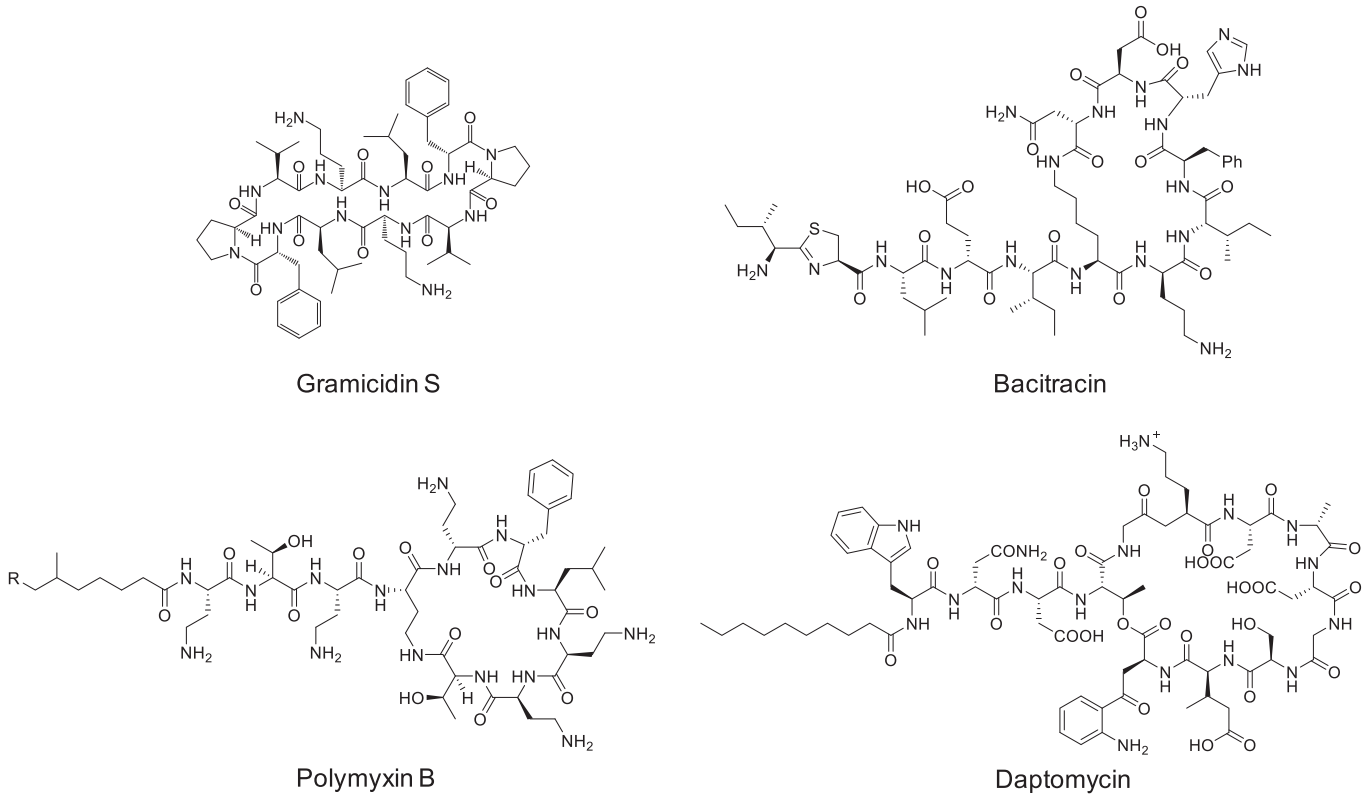


Fig. 6. Structure of gramicidin S, bacitracin, polymyxin B, and daptomycin.

Table 2
AMPs and SMAMPs under phase II-III clinical trials.

No.	Compounds	Company	Prototype	Intended use	Administration	Status/result
1	Pexiganan (MSI 78) [147]	Dipexium Pharmaceuticals	Magainin derivative	Diabetic foot infection	Topical	Phase III completed in 2016/Failed to gain FDA approval
2	Omiganan (MBI-226) [148]	Cutanea Life Sciences, Inc.	Indolicidin derivative	Seborrheic dermatitis	Topical	Phase II ongoing
				Rosacea	Topical	Phase III completed in 2017/Unpublished
				Genital warts	Topical	Phase II completed in 2017/Unpublished
				Catheter-related infection	Topical	Phase III completed in 2008/Failed to gain FDA approval
3	Iseganan (IB-367) [149]	Intrabiotics Pharmaceuticals	Protegrin I derivative	Oral mucositis	Mouth rinse	Phase III completed in 2004/Failed to gain FDA approval
				Ventilator-associated pneumonia	Inhale	Phase III completed in 2005/Failed to gain FDA approval
4	PAC113 [150]	Pacgen Biopharmaceuticals	Histatin derivative	Oral candidiasis	Mouth rinse	Phase II completed in 2008/Unpublished
5	hLF1–11 [151]	AM-Pharma	Lactoferricin derivative	Bacterial Infections and mycoses	Intravenous	Phase I/II completed in 2006
				Candidaemia, bacteremia, and fungal infection	Intravenous	Three Phase I trials withdrawn
6	DPK-060 [152]	ProMore Pharma	Kininogen derivative	Acute external otitis	Ear drops	Phase II completed on 2012/No phase III planned
7	XMP 629 [137]	Xoma Ltd.	BPI protein derivative	Impetigo	Topical	Phase III completed/Failed to gain FDA approval
8	Neuprex (rBPI21) [137]	Xoma Ltd.	BPI protein derivative	Pediatric meningococcemia	Intravenous	Phase III completed/Failed to gain FDA approval
9	CZEN-002 [138]	Zengen	α-MSH derivative	Vulvovaginal candidiasis	Topical	Phase IIb completed in 2004/Positive results announced, but no follow-up trial
10	OP-145 [138]	OctoPlus	LL-37 derivative	Chronic middle ear infection	Ear drops	Phase II completed in 2008/Positive results announced, but no follow-up trial
11	Surotomycin [153]	Cubist Pharmaceuticals/Merck	Daptomycin derivative	*C. difficile* associated diarrhea	Oral	Phase III completed in 2015/Failed to gain FDA approval
12	Delmitide (RDP58) [138]	Genzyme	D-amino acid derivative of HLA	Inflammatory bowel disease	Topical	Phase II completed/Positive result announced, but no phase III planned
13	Ghrelin [154]	University of Miyazaki, Japan	Endogenous HDP	Chronic respiratory failure	Intravenous	Phase II completed/Positive results announced, but no follow-up trial
14	D2A21 [137,155]	Demegen	Synthetic peptide	Burns and wounds infections	Topical	Phase III ongoing
15	Novexatin (NP213) [141]	Novabiotics	Synthetic cyclic peptide	Fungal nail infection	Topical	Phase IIb completed in 2018/Positive results
16	Brilacidin (PMX-30063) [156]	PolyMedix/Cellceutix	Synthetic arylamide oligomer	Skin infections oral mucositis	Topical Mouth rinse	Phase II completed in 2014/Positive results Phase II completed in 2017/Unpublished
17	Lytixar (LTX-109) [157]	Lytix Biopharma AS	Synthetic peptidomimetic	Skin infections	Topical	Phase II completed in 2014/Pending for phase III
18	Exeporfinium chloride (XF-73) [158]	Destiny Pharma	Synthetic di-cationic porphyrin	*Staphylococcal* nasal infections	Topical	Phase II ongoing

phase III trials (entry 14–15). Finally, Synthetic arylamide derivative, peptidomimetic and cationic porphyrin derivative were also found in phase II and III clinical trials (entry 16–18). Although most of these drug candidates have the well-known membrane disruptive antimicrobial mechanism, some of them are also found to act *via* other mechanisms, such as immunomodulation (delmitide and ghrelin) [135,136], disruption of cAMP signalling pathways (CZEN-002) [137], and endotoxin-neutralization (XMP-629) [137].

The derivatives of natural AMPs (entry 1–12) in clinical trials have been extensively reviewed in several reviews [137–140]. Here we only briefly discuss four SMAMPs that have successfully entered phase II trials, including synthetic cyclic peptide novexatin (NP-213, entry 15), arylamide brilacidin (PMX-30063, entry 16), peptidomimetic lytixar (LTX-109, entry 17) and di-cationic exeporfinium chloride (XF-73, entry 18) (Fig. 7). Novexatin is a synthetic cyclic peptide developed by Novabiotics and it contains seven arginine residues [141]. It rapidly kills fungi by lysing their outer membrane [137,140]. A phase II clinical trial using novexatin for the treatment of fungal nail infections by topical administration has been completed in 2018 and promising results were reported. Novabiotics is now preparing for the next stage of development [141].

Lytixar is a synthetic peptidomimetic consisting of one modified tryptophan flanked by two arginine residues. The arginine residues provide the cationic charge, while the *tert*-butyl groups on the tryptophan residue and the phenylethyl group on the modified arginine residue increase the overall hydrophobicity. Lytixar is active against a broad range of Gram-negative and Gram-positive bacteria by disrupting their membrane structure [142]. Lytixar has completed phase II trials for the treatment of impetigo in 2014 and skin infection in 2011 [33]. Promising results were reported, but phase III trial has not been scheduled since 2014.

Brilacidin is arylamide mimic of AMPs. It shows potent antimicrobial activity against both Gram-negative and Gram-positive bacteria by disrupting their cell membrane [143]. Brilacidin was first developed by Polymedix Inc. and later purchased by Cellceutix Corp. A phase II trial using brilacidin for the treatment of *S. aureus* skin infection was completed in 2014 and similar efficacy to daptomycin was reported [33]. Another phase II trial for the treatment of oral mucositis was completed in 2017 and the results showed that brilacidin has a high potential for preventative treatment, as evidenced by a clear reduction of severe oral mucositis (SOM) among patients on brilacidin as compared to those on placebo [144].

Exeporfinium chloride (XF-73) is a synthetic di-cationic porphyrin derivative developed by Destiny Pharma. It contains two cationic ammoniums and one porphyrin core. XF-73 kills microbes through membrane-active antimicrobial mechanism. It is active against a wide range of microbes including Gram-negative, Gram-positive bacteria, and some fungi [145,146]. In particular, it exhibits potent, nonlytic,

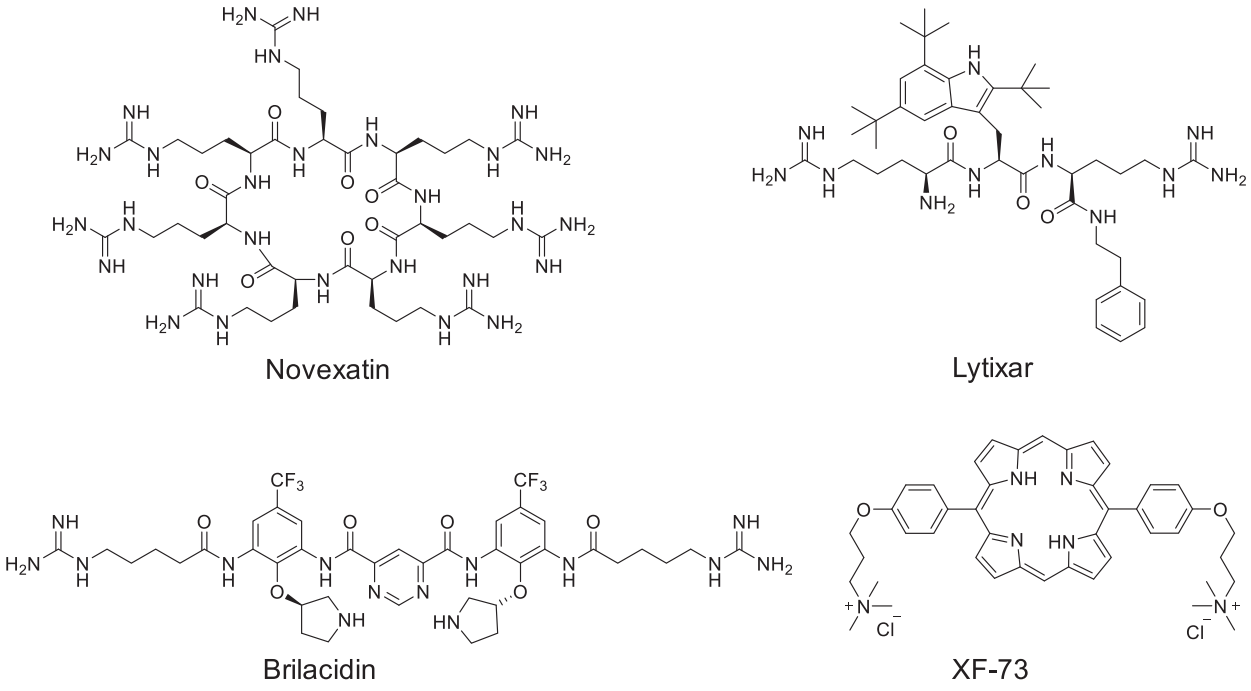


Fig. 7. Structures of synthetic cyclic peptide novexatin (NP-213), peptidomimetic lytixar (LTX-109), arylamide brilacidin (PMX-30063) and di-cationic exeporfinium chloride (XF-73).

bactericidal activity against *S. aureus*. A recent phase II trial using XF-3 for the prevention of post-surgical *Staphylococcal* nasal infections is ongoing.

5. Challenges in the clinical translational development of AMPs and SMAMPs

Despite the extensive studies in the past 30 years and the tremendous progresses achieved, few, if any, AMPs or SMAMPs developed in the second wave of AMP research has been approved for clinical application. There are many challenges slowing down the clinical translation of AMPs and SMAMPs, including low *in vivo* efficacy, high toxicity and financial challenges.

5.1. Low in vivo efficacy

One major dilemma that has impeded the translational development of AMPs is the mismatch between the *in vivo* and *in vitro* antimicrobial efficacy. Despite many AMPs and SMAMPs have demonstrated potent *in vitro* antimicrobial activity, their *in vivo* activity is often questionable [159]. This is especially true in the cases of iseganan, omiganan, pexiganan, surotomycin, neuprex and XMP-629, which have failed the phase III clinical trial due to low efficacy or no advantage compared to an existing drug [137–139]. Many factors account for the low *in vivo* efficacy and they are often associated with the same structure that gives the antimicrobial activity. Currently, most of AMPs are designed to adopt a facially amphiphilic (FA) structure, where the hydrophobic face is separated from the cationic and hydrophilic face. Although this FA structure is critical for bacterial membrane disruption [44], it also causes extensive nonspecific interactions. Many biomolecules, such as serum proteins, DNA, mucins, and glycolipids, in the human body are prevalently negatively charged. Cationic AMPs and SMAMPs tend to non-specifically absorb to these negatively charged biomolecules or cell surface before they can interact with pathogens, thereby significantly limiting the effective concentration [160,161]. Similarly, the hydrophobic face of AMPs can also induce non-specific hydrophobic aggregation with biomolecules and cell surface [162]. Moreover, as most of the AMPs in clinical trials are derived from natural AMPs and consisted of natural L-amino acids with short side chains, they are in general vulnerable to protease degradation [163]. The *in vivo* instability significantly impacts their PK/PD and eventually *in vivo* efficacy.

5.2. Toxicity

Another major obstacle for the AMP clinical translation is the toxicity. The toxicity of AMPs can occur at different levels, including cellular toxicity and systemic toxicity [33]. The cellular toxicity is usually originated from the same cationic and amphiphilic structure that gives antimicrobial activity. Despite less negatively charged lipids are found in the outer leaflet of mammalian membrane compared with bacterial membrane, the mammalian membrane is usually modified with negatively charged glycoproteins and polysaccharides [164], which can help cationic AMPs land onto the mammalian membrane and results in membrane disruption [165]. In fact, many AMPs and SMAMPs are hemolytic when concentration passes certain threshold, limiting their therapeutic index [138]. Alternatively, some AMPs can also bind to cell surface receptors and interfere with the normal signalling pathways, and others can cross the membrane to interact with intracellular proteins, DNAs and organelles, thereby inhibiting protein synthesis and cell proliferation, or inducing cell apoptosis [166,167]. The systemic toxicity is more difficult to predict as the underlying mechanisms are complex. Systemic toxicity can arise from various effects such as undesired immune response, interference of the central nerve system after crossing the blood brain barrier, and blockage blood vessels by inducing blood coagulation [33,130]. Therefore, it is not surprising to see that the majority of AMPs and SMAMPs in clinical trials are designed for topical use only, which is intended to avoid the unpredicted systemic toxicity. Meanwhile, topical application can also increase the local effective concentration and decrease the chance of protease degradation.

5.3. Financial challenge

The financial challenge has also significantly slowed down the development of AMP antibiotics. First, it is usually not impossible at the current stage to find alternative antibiotic therapies from the current

available drug library to combat the drug-resistant microbes. For example, even though MRSA is resistant to methicillin, it is still sensitive to vancomycin, rifampin and fluoroquinolones. Considering the high cost required for new drug development and the competition from existing antibiotics, the profit margin of newly developed antibiotics is usually not very attractive. Consequently, many major pharmaceutical companies like Novartis have dropped their antibiotic research department. Second, instead of going through the expensive and high risky preclinical and clinical studies required for the development of new AMP antibiotics, many pharmaceutical companies tend to develop derivatives or combinations of commercialized antibiotics with known safety and PK/PD profiles, as such an approach is more fall-safe and cost-effective. Third, the cost for producing AMP antibiotics is usually significantly higher than conventional antibiotics, making them less affordable and competitive even if successfully marketed. Finally, the negative results of multiple late-stage clinical trials of AMP antibiotics have dampened the enthusiasm of investors. The insufficient investment further exacerbated the situation.

6. Recent advances in redesign and repurposing AMPs

The challenges in the translational development of AMPs or SMAMPs also triggered many efforts to design new strategies to overcome these hurdles. In this section, the recent progresses in the redesign and repurposing AMPs and SMAMPs to improve their translational potential are discussed. These new strategies have opened many new possibilities and may bring safer and more effective drug candidates with high clinical translational potential.

6.1. New AMP secondary structure design

Currently, most of AMPs and SMAMPs are designed to adopt a FA structure. However, as has been discussed, the exposed hydrophobic domain can induce non-selective aggregation with biomolecules and cell surface, which not only decreases the effective concentration, but also causes toxicity. Moreover, these peptides usually have exposed backbones that are vulnerable to protease degradation. To address these problems, Cheng, Wong, Ferguson and co-workers designed a new class of helical antimicrobial polypeptides adopting a radially amphiphilic (RA) structure, in which the peptide backbone is covered by the long hydrophobic side chains that are terminated with cationic groups [110]. The polypeptides render a radially amphiphilic conformation when viewed from the top/bottom of the helical rod (Fig. 8a). In such design, the helical structure was stabilized by balancing the side-chain charge repulsion with the hydrophobic interaction [168,169]. Because the backbone is well protected by the long side chains, this class of polypeptides exhibit excellent enzyme stability. Meanwhile, these polypeptides also have minimal hydrophobicity-associated cytotoxicity due to the sheltering effect of hydrophilic groups. More interestingly, this helical structure was shown to be critical for antimicrobial activity, as the non-helical counterpart synthesized from the racemic D- and L-amino acids exhibits much lower antimicrobial activity compared to the helical polypeptide. Another strategy to tackle the problems associated with the exposed cationic charge and hydrophobic domain developed a series of single-chain polymeric nanoparticles (SCPNs, Fig. 8b) [170]. In these SCPNs, the hydrophobic groups aggregate in the core and stabilize the nanoparticles, while the cationic groups are distributed on the particle shell. In addition, the surface of nanoparticles is covered by hydrophilic residues functionalized with oligo(ethylene glycol), which can further prevent the non-specific charge-induced aggregation with the negatively charged biomolecules. Decent antimicrobial activity against *P. aeruginosa* PAO1 and its biofilm, and low cell toxicity against human red blood cells were demonstrated. Moreover, these SCPNs exhibited good stability in the presence of negative charged proteins. Modulating the secondary structure of AMPs and SMAMPs could offer a solution to the activity/toxicity paradox [171].

6.2. Smart antimicrobial polypeptides with infection-responsive activity

In mammals, the level of HDPs is regulated in response to bacterial infection [172]. At normal physiological condition, many HDPs are maintained at low level. However, when bacterial infection is detected, the concentration of HDPs is drastically increased either *via* triggered expression or elevated releasing from an inactive protein precursor [172–174]. This infection-responsive mechanism significantly reduces the non-specific toxicity of HDPs. Inspired by this infection-responsive mechanism, several smart antimicrobial polypeptides and polymers have been developed and their activity can be activated in response to bacterial infection or their microenvironment [111,112,175]. Cheng, Yin and co-workers designed a class of antimicrobial polypeptides that

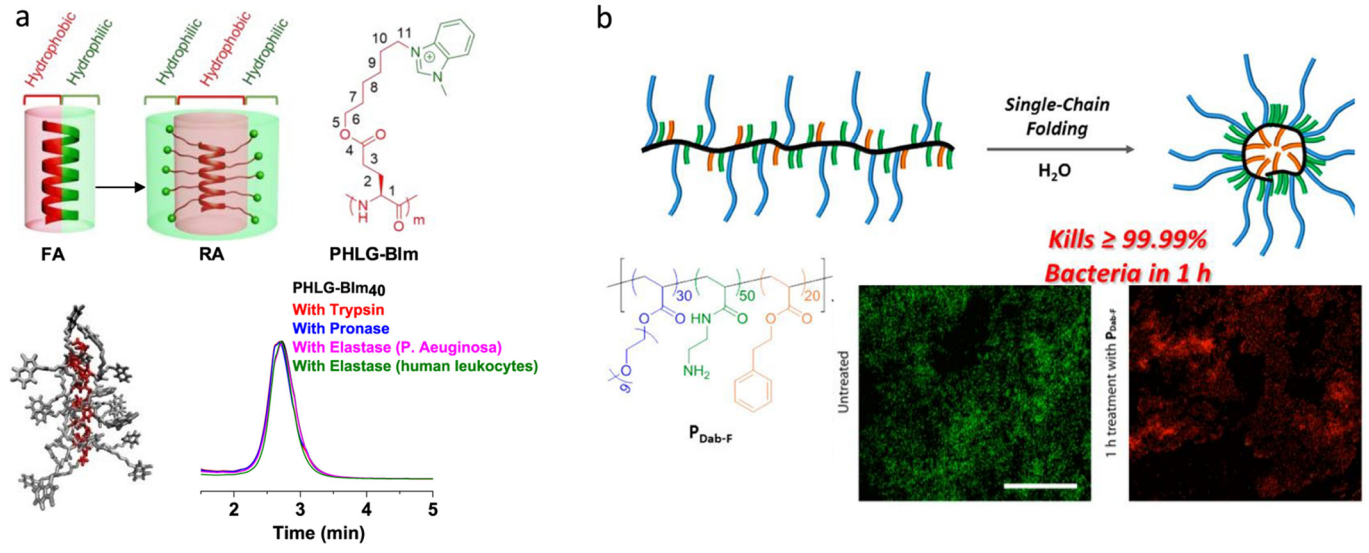


Fig. 8. Two representative strategies of redesign AMPs and SMAMPs. (a) Facially amphiphilic (FA) and radially amphiphilic (RA) AMPs. The chemical structure and the simulated 3D conformation of a RA-AMP, PHLG-Blm, are shown. This RA-AMP has high enzyme stability, as demonstrated by the SEC trace after enzyme treatment. Adapted with permission from [110]. Copyright 2015 National Academy of Sciences. (b) Single-chain polymeric nanoparticles (SCPNs) with sheltered cationic charge and hydrophobic side chains. The structure of a representative SCPN and its antimicrobial activity against *P. aeruginosa* (PAO1) biofilm are shown. Reprinted with permission from [170]. Copyright 2017 American Chemical Society.

can be activated by bacterial phosphatase (Fig. 9a) [112]. These polypeptides contain both cationic residues and anionic phosphorylated phenylalanine residues. Under normal condition, the electrostatic attraction between the phosphate and the cationic ammonium drives the polypeptides to adopt a random coil conformation and the polypeptides are neither active to bacteria nor toxicity to human cells. However, when bacterial infection happens, the bacterial phosphatase cleaves the phosphate from the phenylalanine residues and the polypeptides change to a helical conformation with high membrane activity, which can then actively kill bacteria by disrupting their cell membrane.

In another work, the same group designed a different type of pH-activated coil-to-helix switchable polypeptides for selective eradication of *H. pylori*, a pathogen that thrives in the unique acidic environment of stomach and infects ~50% of world population [176,177]. These pH-activated polypeptides contain both glutamic acid residues and cationic residues functionalized with hydrophobic moieties (Fig. 9b) [111]. In the physiological neutral pH, the electrostatic attraction between cationic residues and the anionic residues distorts the helical structure and the polypeptides have low toxicity to human cells as well as to commensal bacteria in intestine, which play an important role in human gut health [178]. However, when the polypeptides enter the stomach with a pH ~1–3, the protonation of glutamic acid quenches the electrostatic attraction, and the polypeptides resume to the helical conformation. This helical conformation exhibits high membrane activity against bacteria and can selectively kill *H. pylori* in stomach without hurting the commensal bacteria in the intestine. In fact, Jiang, Yang and co-workers have also previously reported another type of acid-activated antimicrobial poly(methacrylate) derivatives that can actively kill both Gram-negative and Gram-positive bacteria in acidic pH 5.0 [175].

6.3. Nano-antimicrobial

Inspired by the multivalent interactions found ubiquitously in nature, there has been a growing interest recently in the development of antimicrobial nanoparticles, namely “nano-antimicrobials”, base on the rationale that enhanced antimicrobial activity can be achieved by increasing the multivalency of physical interactions [179,180]. This rationale is further supported by the membrane penetrating mechanisms of AMPs, in which the cooperation of multiple peptide chains is usually required for punching a pore on bacterial membrane. In early works, Yang, Li, Hedrick and co-workers reported a series of antimicrobial micelles self-assembled from amphiphilic antimicrobial peptides or polymers [181–185], with the intention to increase the local peptide concentration and intensify the charge interaction. One representative antimicrobial micelle is a self-assembly of an amphiphilic peptide composed of cholesterol, glycine, arginine and HIV TAT sequence (Fig. 10a) [184]. They found that the self-assembled nanoparticles exhibit much higher antimicrobial activity against bacteria and fungi than their unassembled peptide counterparts. They can even cross the blood-brain barrier to kill *S. aureus* that causing meningitis. Meanwhile, the toxicity of these nanoparticles is not significantly changed compared to their building blocks, which significantly increases their therapeutic index. Interestingly, the mechanistic studies suggest that these nanoparticles not only disrupt bacterial membrane, but also damage the bacterial cell wall [184]. These pioneer studies indicated that nanostructure could be used to increase the local concentration of membrane-active antimicrobials and is another important factor to be considered for the optimization of antimicrobial activity and selectivity. Since then, various self-assembled antimicrobial nanoparticles have been reported [179,180,186–188]. Later, Qiao, Reynolds and co-workers reported a different class of antimicrobial nanoparticles that are constructed by covalent bonding and termed them as ‘structurally nanoengineered antimicrobial peptide polymers’ (SNAPPs) [189]. SNAPPs were prepared by using polyamidoamine (PAMAM) dendrimers as the core to grow random co-polypeptides of lysine and valine by NCA-ROP method (Fig. 10b). SNAPPs can actively kill the recalcitrant multidrug-resistant Gram-negative bacteria *A. baumannii* both *in vivo* and *in vitro*, with activity comparable to or higher than antibiotic imipenem. They have low cytotoxicity to human cells and good therapeutic index (range from 50 to 170). Moreover, they induced much lower drug resistance than imipenem when

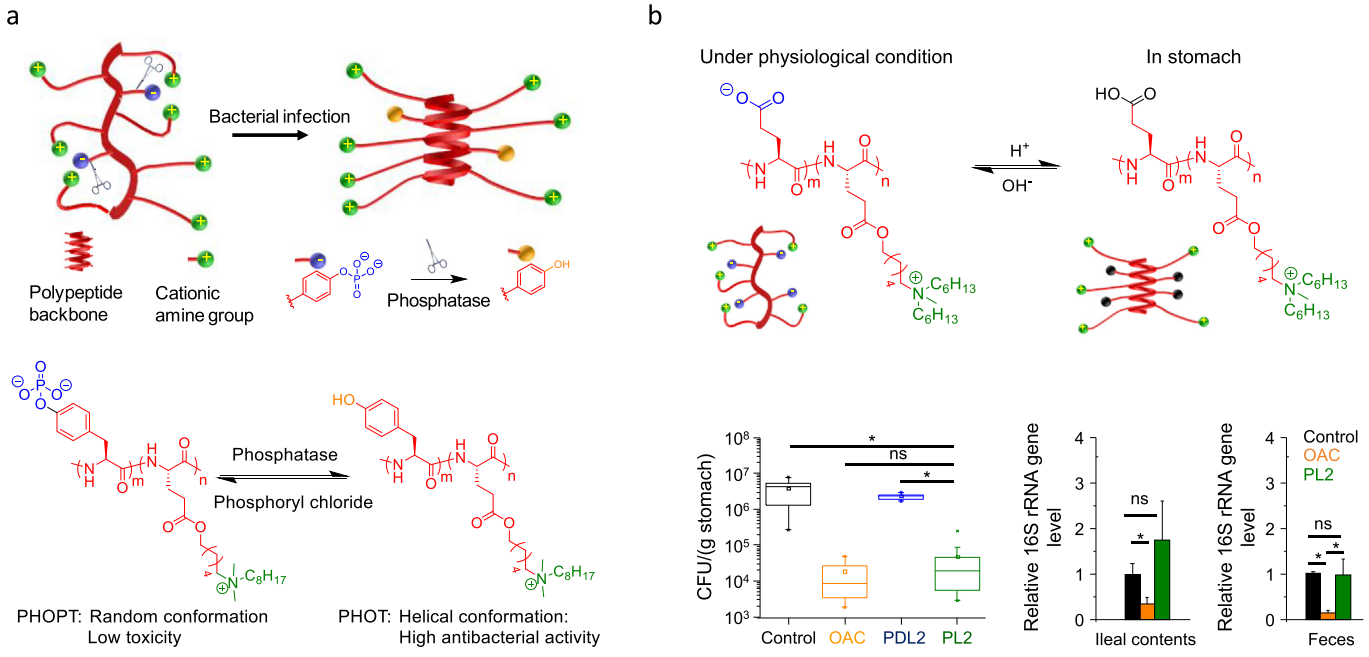


Fig. 9. Smart antimicrobial polypeptides with infection-responsive activity. (a) Schematic representation and the structure of antimicrobial polypeptides that can be activated by bacterial phosphatase. After cleavage of the phosphates by bacterial phosphatase, the polypeptides change from a low activity state to high activity state. Adapted with permission from [112]. Copyright 2017 John Wiley and Sons. (b) pH-activated antimicrobial polypeptides for selective eradication of *H. pylori* in stomach, with minimized toxicity against commensal bacteria in ileal and fecal contents. OAC, the triple therapy (omeprazole/amoxillin/clarithromycin) used in clinical; PL2, pH-activated coil-to-helix switchable polypeptide; PDL2, the racemic counterpart of PL2 synthesized from D- and L-amino acids. PDL2 is unable to undergo pH-activated coil-to-helix transition. Adapted with permission from [111]. Copyright 2017 National Academy of Sciences.

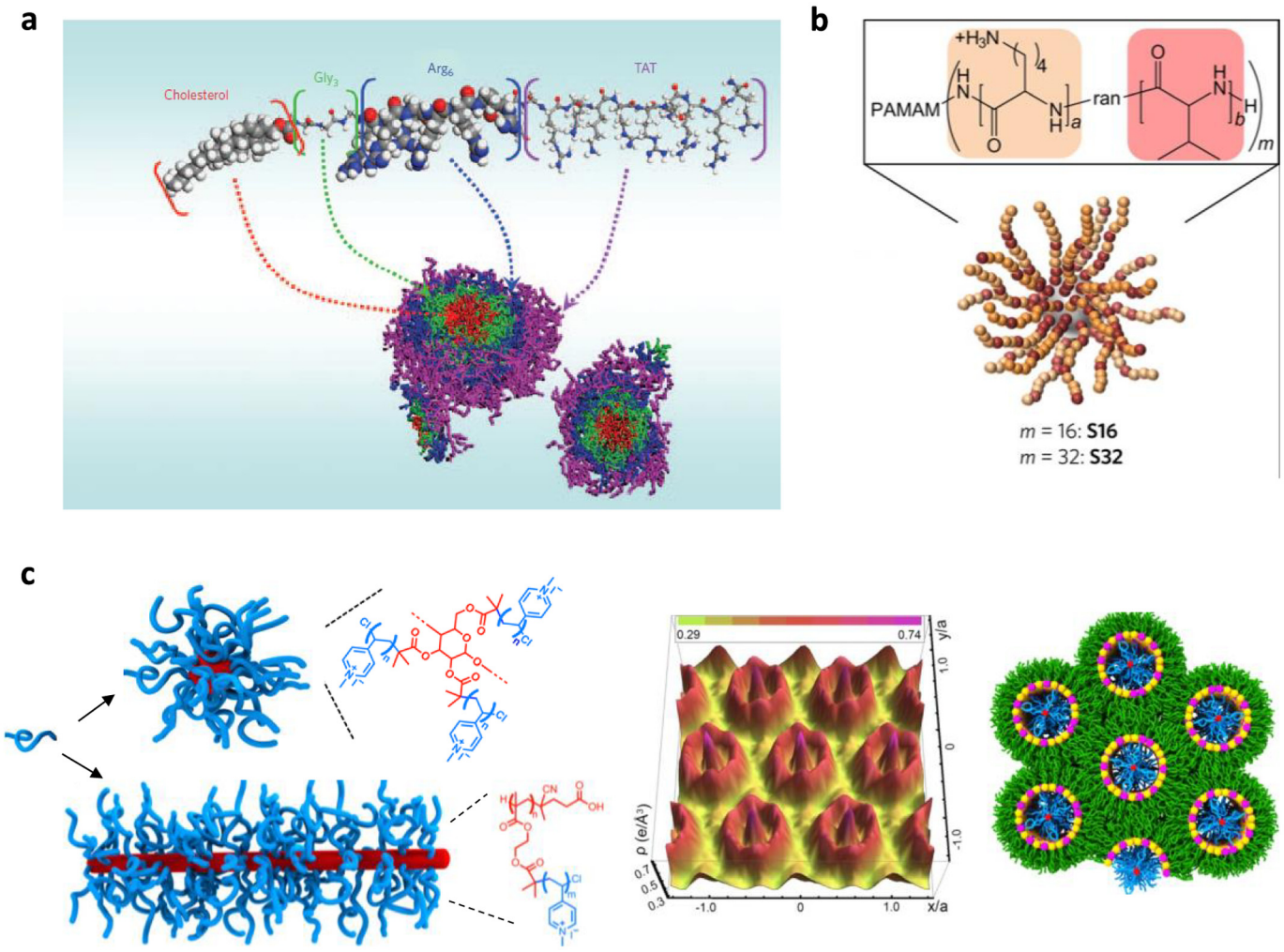


Fig. 10. Nano-antimicrobials constructed from antimicrobial peptides and polymers. (a) Antimicrobial micelle self-assembled from an amphiphilic cationic peptide composed of cholesterol, glycine, arginine and TAT. Reprinted with permission from [184]. Copyright 2009 Springer Nature. (b) Structurally nanoengineered antimicrobial peptide polymers (SNAPPs) synthesized by the surface-initiated ring-opening polymerization of lysine and valine NCAs. Reprinted with permission from [189]. Copyright 2016 Springer Nature. (c) Hydrophilic and cationic polymer molecular brushes (PMBs) of different nanostructures revealed nanostructure-dependent antimicrobial mechanism. Left: the structure of spherical PMB and rod-like PMB; middle: the electron density map of a bacteria-mimicking membrane treated with rod-like PMB, reconstructed from its small angle x-ray scattering profile; right: the model of an inverted hexagonal phase uncovered by the electron density map. Adapted with permission from [190]. Copyright 2017 American Chemical Society.

used in sub-inhibitory concentration. They kill bacteria by disintegrating the bacterial cell wall and cell membrane. Nevertheless, despite various nano-antimicrobials have been reported, a clear SAR has not been established. Recently, Jiang, Liang and co-workers studied the structure-activity of phage-mimicking polymer molecular brushes (PMBs) of different nanostructures (Fig. 10c) [190]. Using an inactive hydrophilic linear polymer as the building block, they constructed model PMBs with well-defined spherical or rod-like shapes [190]. They showed that the nanostructure can change the initially inactive linear polymer to highly active nano-antimicrobials. Moreover, they also found that the antimicrobial activity of PMBs varies with their nanostructures. While the spherical and short rod-like PMBs are active to both Gram-negative and Gram-positive bacteria, the long rod-like PMB is selectively active to Gram-negative bacteria. This antimicrobial specificity is attributed to the difference in the cell wall and cell membrane structures between Gram-positive and Gram-negative bacteria. The peptidoglycan cell wall of Gram-positive bacteria has a mesh size of diameter 5–50 nm [191], which is too small to give the free access of long rod-like PMB (diameter ~ 7 nm, length ~ 70 nm) to the bacterial cell membrane. More interestingly, they uncovered that the nanostructure can change the way how the molecule interacts with bacterial membrane. While the linear chain hydrophilic polymer induces bacteria membrane to form cubic phases, the nanoparticles were found to induce the membrane to form porous inverted hexagonal phase. Although exciting progresses have been made in nano-antimicrobials, the research in this area is still in a very early stage. The *in vivo* safety, efficacy, bioavailability, and PK/PD of nano-antimicrobials remain to be extensively investigated before any possible clinical application.

6.4. Small molecular SMAMPs

As mentioned in Section 3.1, although synthetic peptides and peptide mimics have higher stability and sometimes superior activity and selectivity compared to many natural AMPs, their application was still limited by the multistep synthetic process and low overall yield due to their relatively large molecular weight. Further limitations also include the poor bioavailability and PK/PD profile resulted from the strong non-specific charge interactions with biomolecules. Recently, small SMAMPs (Mw <1000) have emerged as a class of interesting antimicrobial agents that can be potentially explored as an alternative to large SMAMPs (Mw > 1000) [192–194]. Although highly condensed in structure, some small SMAMPs have potent antimicrobial activity and excellent selectivity. There are many reasons why small SMAMPs are preferred: first, they are relatively simple to synthesize, which can

significantly lower the cost and make the large-scale production more accessible [192]. Second, their low-valent charge interaction with biomolecules is completely reversible, so that their PK/PD profile is comparable to other low charged small-molecule drugs. Third, they are monodispersed small molecules with defined metabolites, which can avoid the problems associated with the heterogeneous polymeric antimicrobials. Moreover, they can pass easily into the bloodstream through the intestinal wall. From the bloodstream, they can reach almost anywhere in the body [192]. This property could possibly be used for systemic application. Many different small SMAMPs have been reported (Fig. 11). In early works, Tew, Degrado and coworkers developed different series of small SMAMPs with different degree of backbone rigidity based on the derivatives of arylamide [105,195], urea [196] and phenyleneethylene [39,197]. These small SMAMPs have very simple structure and can be synthesized in very few steps with high yield. Although their initial intention was to study the role of rigidity in antimicrobial activity, they found that the amphiphilicity indeed plays a more dominant role than rigidity. By tuning the amphiphilicity, some small SMAMPs achieved excellent activity and selectivity. Optimized phenyleneethylene derivative AMO2 (Fig. 11), for example, have a MIC less than 2 μg/mL against both Gram-negative and Gram-positive bacteria, and a selectivity larger than 90 [39]. In fact, the previous discussed brilacidin is an optimized arylamide derivative developed by this group. Cai on the other hand, reported several different types of small SMAMPs based on hydantoin [198], acylated reduced amide [199], biscyclic guanidine [200], and dimeric alkylamides of lysine [193,201]. These small SMAMPs can be synthesized easily. By optimizing the amphiphilicity, molecules with very high antimicrobial activity were obtained. For example, the hydantoin derivative shown in Fig. 11 has a MIC <1 μg/mL against both Gram-negative (*E. coli* and *P. aeruginosa*) and Gram-positive bacteria (MRSA and VRE). More interestingly, its *in vivo* efficacy outperforms vancomycin in a rat model bearing MRSA pneumonia [198]. These small SMAMPs have high translational potential. Strøm and coworkers reported a group of small SMAMPs based on $\beta^{2,2}$-amino acid derivatives [202–204]. Through well-designed structure-activity studies, they identify several small SMAMPs that fulfill the Lipinski's rule of five: (1) the octanol-water partition coefficient logP should be less than 5; (2) the Mw should not exceed 500 Da; (3) a maximum of 5 hydrogen bond donors; and (4) no more than 10 hydrogen bond acceptors. [202]. These $\beta^{2,2}$-amino acid derivatives demonstrate good *in vitro* activity and selectivity against both Gram-positive and Gram-negative bacteria. Some compounds exhibit good activity against the biofilm formed by *S. aureus* [204]. However, the *in vivo* efficacy, safety, and PK/PD remain to be evaluated before pushing for clinical translation. Many other small SMAMPs have also been reported, including the *tri*-peptide derivatives and lipid derivatives. In fact, two drug candidates currently in clinical trials, LTX-109 and CSA-13, are the derivatives of *tri*-peptide and lipid [205,206], respectively. The small SMAMPs have been extensively reviewed elsewhere [192–194].

6.5. In combination with antibiotics

Given the challenges encountered in the clinical translation, a different opinion that AMPs/SMAMPs should be used as a component in antibiotic combination therapy, instead of as the single active ingredient, has received significant attention in recent years. Combination therapy is being used widely in the treatment of many health conditions and has recently been regarded as a promising and cost-effective solution for bacterial infections to overcome the inadequacies of antibiotic monotherapy [207]. Moreover, as many bacteria evade the action of antibiotics by limiting their intracellular access and accumulation, the membrane-active AMPs are particularly attractive to be used synergistically with antibiotics. Currently, two different strategies have been

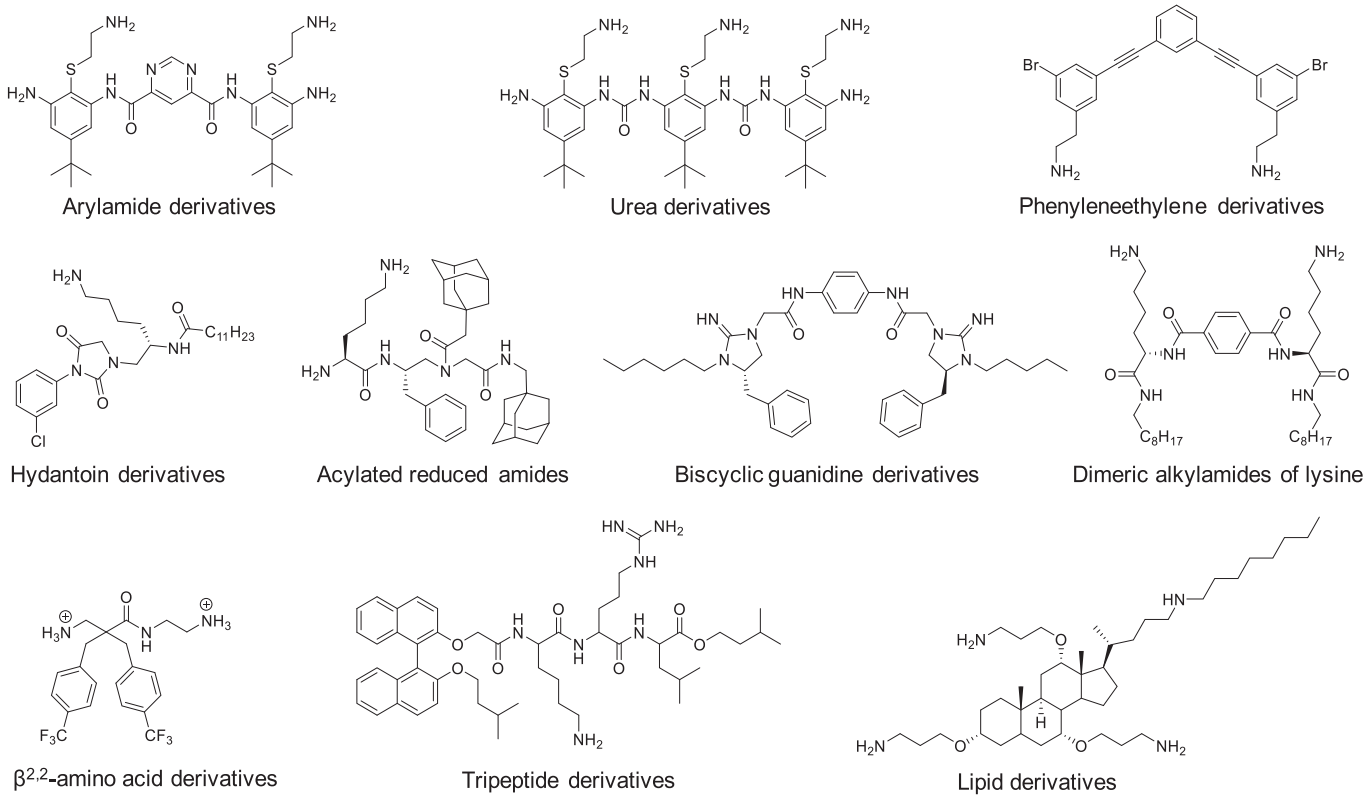


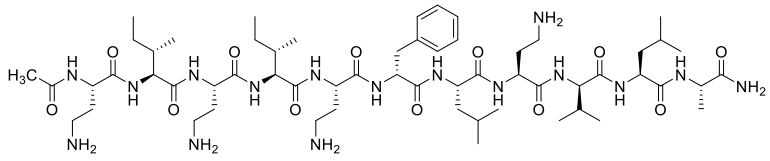
Fig. 11. Representative structure of small SMAMPs based on the derivatives of arylamide, urea, phenyleneethylene, hydantoin, acylated reduced amide, biscyclic guanidine, and dimeric alkylamides of lysine, $\beta^{2,2}$-amino acid, *tri*-peptide and lipid.

developed to combine the function of AMPs with antibiotics. In the most straightforward strategy, AMPs are directly mixed with antibiotics and used as combination therapies to kill the multidrug-resistant bacteria. Many different antibiotic/AMP combinations have been tested and the majority of them exhibited a positive synergistic effect [208–213]. In one example (Fig. 12a), Zhu, Shen and coworkers recently reported that a short linear cationic AMP (SLAP)-S25 is able to restore the activity of cefepime, colistin, ofloxacin, rifampicin, tetracycline and vancomycin against a panel of multidrug-resistant Gram-negative pathogens [208]. The mechanism studies suggest that S25 triggers membrane damage by binding to the LPS in the outer membrane and the phosphatidylglycerol in bacterial cytoplasmic membrane, thereby increasing the intracellular access of these conventional antibiotics and potentiating their antimicrobial efficacy [208]. In fact, the efficacy of this combination strategy has been verified in multiple clinical settings. Notably, the combination of AMP colistin with other antibiotics significantly reduced the mortality in patients infected with *Klebsiella KPC*, as compared to patients treated with monotherapy [214,215]. The other strategy is to attach antibiotics to AMPs *via* a cleavable or non-cleavable covalent bond to develop antibiotic-AMP conjugates. For example, Cegelski, Wender and coworkers conjugated vancomycin to octaarginine (r8) *via* amidation between the *N*-terminal amine of r8 and the carboxyl group of vancomycin (Fig. 12b) [216]. The conjugate (V-r8) effectively eradicated biofilm formed by *S. aureus* and was more active than vancomycin, octaarginine or the 1:1 mixture of vancomycin and octaarginine. Various conjugates have also been developed by different coupling chemistry [217,218]. However, the synergistic effect varies. While some conjugates were reported to have positive synergistic effects [219–221], others were reported to have either no or even negative synergistic effects [211]. To achieve optimal synergistic effects, factors such as modification sites, linker cleavability and flexibility and conjugation chemistry need to be carefully explored in each different case [217].

7. Summary and perspective

The antibiotic resistance is an ongoing issue and is projected to continuously deteriorate in the future. More efforts and investments sustaining the development of new antimicrobial agents are needed to protect public health [222]. AMPs are a class of attractive candidates as the next generation of antibiotics. With the unique membrane-disruptive and multi-targeting antimicrobial mechanism, they can actively kill antibiotic-resistant microbes and are difficult for microbes to develop drug resistance. However, it has been reported that bacteria have evolved several mechanisms to attenuate the action of natural AMPs [10,44]. One major mechanism is to reduce the net negative surface charge by modifying structure and composition of surface polysaccharides or membrane lipids and thereby hindering the peptide attachment [223,224]. Another mechanism is to secret proteases to degrade and deactivate AMPs [44]. For example, LL-37 is cleaved and inactivated by an *S. aureus* metalloproteinase named aureolysin [163]. Despite these resistance mechanisms, widespread resistance to AMPs is rare compared to conventional antibiotics. Besides, some of these resistance mechanisms can be circumvented through engineering approaches, as has been demonstrated by SMAMPs. With a non-natural origin, SMAMPs have been shown to be resistant to protease

a



Antibiotic	MIC[a] (μg ml^{-1})	FIC index	MIC[b] with S25 (μg ml^{-1})
Cefepime	32	0.156	1
Colistin	8	0.047	0.25
Ofloxacin	32	0.094	2
Rifampicin	128	0.031	0.031
Tetracycline	128	0.281	16
Vancomycin	128	0.094	8

b

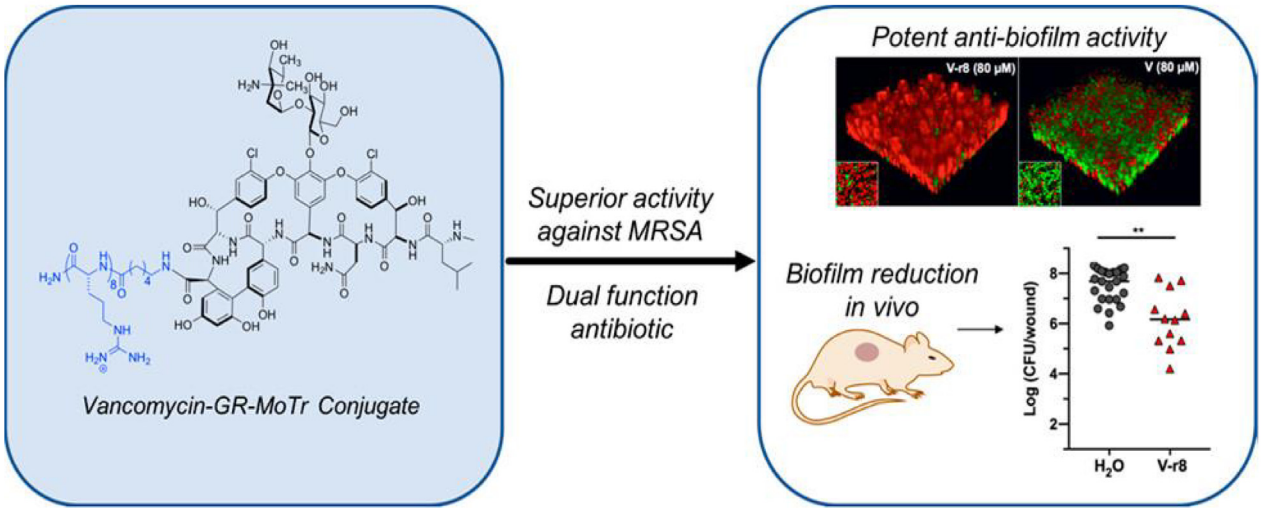


Fig. 12. Combination therapies and antibiotic-AMP conjugates. (a) A representative antimicrobial peptide SLAP-S25 (S25) works synergistically with antibiotics including cefepime, colistin, ofloxacin, rifampicin, tetracycline and vancomycin. The MICs of antibiotics in the absence or presence of SLAP-S25 (4 μg/mL) and fractional inhibitory concentrations (FICs) were shown in the table. FIC < 0.5 indicates the synergistic effect. Adapted with permission from [208]. Copyright 2020 Springer Nature. (b) Vancomycin-D-octaarginine conjugate (V-r8) effectively eradicates biofilm both *in vitro* and *in vivo*, and is more active than vancomycin, octaarginine or the 1:1 mixture of vancomycin and octaarginine. Reprinted with permission from [216]. Copyright 2018 American Chemical Society.

degradation. By controlling the charge density, strong attachment to bacteria with reduced net surface charge can still be realized.

Significant and encouraging progresses have been made in the research aspect of AMPs/SMAMPs, nevertheless, success in the clinical translation has yet to be achieved so far. There are many possible reasons for the slow translation progress. On the one hand, the current design strategies are struggling under the shadow of the activity/toxicity paradox. Antimicrobials with high antimicrobial activity oftentimes have appreciable toxicity that eventually limits their application *in vivo*, especially in systemic application. It is therefore not surprising that most of AMPs/SMAMPs in clinical trials are designed for topical use only. On the other hand, the lack of good models or evaluation methodologies for compound screening may account for the mismatch between the *in vitro* and *in vivo* efficacy. Currently, the general convention for AMPs/SMAMPs screening is to first determine their *in vitro* activity (MIC/MBC) and toxicity (hemolysis and cell viability) before *in vivo* evaluation in animal models. However, the relevance of *in vitro* activity and toxicity to *in vivo* efficacy and safety is largely unknown. The most active and selective compound identified *in vitro* does not necessarily mean the same trend *in vivo*. New effective and more predictive methodologies for compound screening can facilitate the clinical translational development. Furthermore, the compound library (5–500 compounds) obtained from the rational design strategies is usually not inclusive enough to include the optimal drug candidates with high translation potential. Currently, most of SMAMPs reported so far are designed by rational design, a method that usually yields a relatively small compound library. Therefore, new designing strategies, screening methodologies and large compound library can facilitate the clinical translation of AMPs/SMAMPs.

In recent years, creative designing strategies have been actively developing, aiming to address some of the major challenges in the clinical translation of AMPs/SMAMPs. Redesigning the secondary structure of AMPs is an interesting approach to resolve the activity/toxicity paradox. By attenuating or sheltering the structural components that cause toxicity while maintaining the components required for antimicrobial activity, AMPs with high activity, selectivity and therapeutic index are expected to be designed in the near future. The development of smart AMPs that are responsive to bacterial infection or their microenvironment represents another attractive approach to control to activity and toxicity of AMPs. In fact, this approach has been widely used in nature where many AMPs are maintained at low level at normal physiological condition to reduce nonspecific toxicity but are either triggered to express or released from an inactive protein precursor upon detecting bacterial infection. Moreover, the development of nano-antimicrobials could also be a promising direction. By assembling multiple AMPs or polymers into nanoparticles, a single nanoparticle is able to provide a high local concentration required for punching pores on bacterial membrane, without the need of reaching the global effective concentration that is oftentimes toxic. In addition to the enhanced antimicrobial activity, these nanoparticles can also be designed to be multifunctional by incorporating different drugs into these nanoparticles. For example, Du and co-workers designed polymeric micelles with both antimicrobial and anticancer activities by incorporating anticancer drug doxorubicin into these cationic micelles [225]. However, issues such as the toxicity associated with nanostructures, the quality control and the metabolism of the heterogeneous nanoparticles, and the less predictable PK/PD profiles are new emerged challenges that remain to be resolved before the clinical translation of nano-antimicrobials. The small SMAMPs, which have very simple and well-defined structure, are small molecules with high drug potential compared to the large SMAMPs. Their drug potential has been demonstrated by the at least three drug candidates (PMX-30063, LTX-109 and CSA-13) that have entered phase II or III clinical trials. More importantly, their similarity to other small molecular drugs indicates that they could be used for systemic application, which is barely achievable by the large SMAMPs. More small SMAMPs are expected to enter clinical trials and even clinical application in the near future. Finally, the combination of AMPs with antibiotics could be an easy and effective way to reactivate many conventional antibiotics as well as to elongate their lifespan. Two important antibiotic-resistant mechanisms are commonly used by microbes: i) decreasing the membrane permeability to antibiotics, and ii) reducing the intracellular antibiotic accumulation by efflux pump. The membrane activity of AMPs could be the very solution to these antibiotic-resistant mechanisms and can reactivate many antibiotics that have lost their efficacy. More recently, machine learning and database-filtering technology (DFT) have also been employed to efficiently screen sequence space from an established database and guide experiments toward promising candidates with high *in vitro* and *in vivo* activity [226–229]. With these new design strategies, and together with the continuous development of new screening methods and large compound library, more AMPs and SMAMPs are expected to be successfully brought into clinical application in the future. Moreover, their clinical indication may expand from topical application to systemic administration once the activity/toxicity paradox is resolved.

Declaration of Competing Interest

There are no conflicts to declare.

Acknowledgements

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